SPECIFICITY OF ANTIBODIES BINDING ORGANOPHOSPHORUS HAPTENS

by

Abigail Capistrano Buenafe

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To my husband, Jack,

for his love and support throughout this endeavor,

To my parents, Mr. and Mrs. Procopio A. Buenafe,

for their love and encouragement,

To both of our families,

for their support, their inspiration and my sanity.

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ABBREVIATIONS

APSo	*p*-aminophenyl-Soman
Ars	*p*-azophenylarsonate
BSA	bovine serum albumin
CDR	complementarity determining region
CFA	complete Freund's adjuvant
C_H	immunoglobulin heavy chain constant region
D	diversity region
DIMP	diisopropylmethylphosphonate
DPMP	dipinacolylmethylphosphonate
ELISA	enzyme-linked immunosorbent assay
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
J	joining region
KLH	keyhole limpet hemocyanin
M603	McPc603 myeloma
mAb	monoclonal antibody
NP	4-hydroxy-3-iodo-5-nitrophenylacetyl
NPDBP	*p*-nitrophenyl-3,3-dimethylbutylphosphate
NPEP	*p*-nitrophenylethylphosphate
NPMPC	*p*-nitrophenylmethylphosphocholine
NPPC	*p*-nitrophenylphosphocholine
NPSo	*p*-nitrophenyl-Soman
OHSo	hydroxy-Soman
OVA	ovalbumin
PBS	phosphate-buffered saline
PC	phosphocholine
R:S	ratio of replacement to silent mutations
So	Soman [O-1,2,2 trimethylpropylmethylphosphonofluoridate]
TNP	2,4,6-trinitrophenyl
V_H	heavy chain variable region
$V\kappa$	kappa light chain variable region
V_L	light chain variable region
$V\lambda$	lambda light chain variable region

ABSTRACT

The ability of the immune system to recognize and respond efficiently to a diverse and probably limitless array of antigens is indeed impressive. Equally impressive are the intricate mechanisms, uncovered with the aid of molecular technology, that regulate the generation of diversity and yet impart such specificity. The work described here is presented with the hope that it may lend to an understanding of how the specific antibody response matures and is governed by these mechanisms.

The central theme of this study is the fine specificity and molecular characterization of the antibody response to a novel organophosphorus hapten, Soman [O-1,2,2 trimethylpropylmethylphosphonofluoridate], as coupled to the protein carrier, keyhole limpet hemocyanin (KLH). The antibody response to Soman-KLH has not been used as a model for immune maturation prior to the work in this laboratory and, therefore, relatively little was initially known of this specific process. The major strength of the anti-Soman-KLH response as a model system lies in the observation that unique structural features are shared between the protein-coupled form of Soman and that of another organophosphorus hapten, phosphocholine (PC)(described in detail within). An important distinction between the haptens PC and Soman is that while PC is a frequently encountered environmental antigen, Soman is not.

The response to PC has been extensively studied by many groups; the response to PC-KLH in particular has been used in this laboratory for several years as a model system for immune maturation. Previous work has established that a dramatic shift in fine specificity and V gene expression occurs in going from the primary to the secondary anti-PC-KLH response. The

mechanism(s) responsible for this transition clearly involves selection by the immunogen but is not completely understood. We have taken advantage of the structural similarity between Soman-KLH and PC-KLH to ask questions concerning the role of specific molecular interactions in contributing to this selective process. In particular, the phenyl structure which links both Soman and PC to the protein carrier apparently plays a significant role in recognition by anti-Soman-KLH and anti-PC-KLH antibodies. Yet, little cross-reactivity between antibodies specific for these related structures exists and is likely to be related to particular charge differences found in Soman and PC. By comparing the specificity and maturation pathways of these two responses, we therefore have a means of distinguishing between antigenic structures which may be required for binding vs. structures required for binding but which also contribute to non-cross-reactive specificity.

In this work, characterization of the specific antibody response to Soman-KLH is described. Specific objectives in the development of the anti-Soman-KLH response as a model system for studying immune maturation were:

1) To determine the level of cross-reactivity existing between anti-Soman-KLH- and PC-KLH-specific antibodies given the overall structural similarity of Soman-KLH to PC-KLH.

2) To define structural regions of Soman-KLH important for specific recognition.

3) To determine the pattern of V gene usage, restricted vs. heterogeneous, associated with the Soman-KLH-specific response.

4) Definition of structure-function relationship of the Soman-specific combining site with regard to hapten-specific interaction.

LITERATURE REVIEW

Rearrangement of Immunoglobulin Genes

Variable domains of immunoglobulin are encoded by discrete gene segments which are somatically rearranged in a programmed manner during B cell development(1-4). Rearrangement initiates in pro-B cells at the heavy chain (H) locus and involves recombination of a D_H (diversity) segment with a J_H (joining) segment. This is followed by rearrangement of a V_H segment to the DJ_H complex and the expression of a complete IgH transcript containing the sequences of VDJ_H and the Cμ constant region. Although mature B cells often possess rearrangements on both heavy chain alleles, only one heavy chain protein is produced (5,6). The mechanism of allelic exclusion remains obscure but may involve feedback inhibition mediated by the membrane form of the expressed Cμ heavy chain (7,8). Rearrangement of V_L to J at the light chain loci (kappa or lambda) typically follows the expression of Cμ heavy chain, and it has been suggested that the presence of functional Cμ is required for the induction of light chain gene rearrangement (9,10). Furthermore, rearrangement of kappa vs. lambda light chain genes appears to be regulated in most B cells in that kappa-producing B cells are often found with unrearranged lambda light chain genes while lambda-producing B cells are found with non-functionally rearranged or deleted kappa genes (10,11). The implication from these observations is that lambda rearrangement must follow kappa rearrangement, and is initiated only in the event that the kappa rearrangements are non-productive. However, the existence of a B cell subpopulation capable of co-expressing kappa and lambda light chains and

which may not possess this kappa feedback mechanism has also been proposed (12,14).

Further recombination at the heavy chain locus may occur involving rearrangement of C_H gene segments in activated B cells (2,15). This mechanism of class switching juxtaposes the rearranged VDJ segment with the C_H genes located downstream of Cμ via recombination between C_H-associated switch regions (16,17) and results in the deletion of intervening sequences. Another mechanism proposes that class switching may also occur at the level of RNA processing thus leaving all C_H sequences intact (18,19). Class switching appears to be a directed process (20,21) and may be modulated by T cell-derived cytokines (22-24). Also, there appears to be a correlation between class switching and the induction of somatic hypermutation but this does not appear to be an obligate association. The combination of a variable domain with different heavy chain isotypes results in specific antibodies capable of different effector functions. Association with different heavy chain isotypes appears not to affect specificity of the variable domain (25,26,27).

Generation of Antibody Diversity

In the mouse there are four JH, twelve known DH, and an estimated several hundred to thousand VH gene segments which may contribute to the antibody repertoire (28,29,30). Approximately two hundred Vk, four Jk, and three lambda genes are also available (28). It is obvious from this that considerable antibody diversity can be generated from multiple V-D-JH and V-Jk combinations alone. Additional heterogeneity is generated from the recombination process through imprecise joining and through the addition of non-V, D, or J derived nucleotides (N sequences) typically found at the V-D and

D-J joints. Yet another level of diversity is created by the combinatorial association of VH and VL.

VH genes have been classified into at least eleven families on the basis of sequence homology and range in size from two members to greater than one hundred members each (29,30,31). Biased utilization of VH gene families most proximal to the JH locus has been observed in B lineage cells derived from fetal and neonatal BALB/c mice (4,32,33) and has been suggested to reflect developmentally controlled changes in the accessibility of the VH locus to a recombinase (32,34). Such a regulated pattern of V gene expression may explain the ordered appearance of immune responsiveness to certain antigens during ontogeny (1,4). A shift occurs such that VH gene utilization in B cells of adult BALB/c mice correlates more closely with the estimated size of each family reflecting random gene usage (31,35). However, strain differences in the pattern of VH gene usage have been observed (31,36,37) and the actual frequency of functional vs pseudogenes associated with each family is unknown (30). Relatively little is known about utilization of the 29 Vk families although no positional bias for Jk proximal families has been found and the use of certain Vk gene families may not be proportional to genomic complexity (38).

Diversity derived from multiple gene recombination and VH-VL association is contributed in the absence of selection by exogenous antigen and constitutes a potential antibody repertoire. The total potential repertoire has been estimated to consist of $>10^8$ different antibody specificities (28). Somatic hypermutation, another important diversification mechanism, appears to be triggered upon antigen stimulation through specific immunoglobulin receptors on the B cell surface (39,40). The mechanism of this mutational process is unknown. These induced somatic point mutations occur

randomly throughout the V region and have been detected up to approximately 250 bp upstream of the V coding region (41). Moreover, these mutations appear to be associated more with VH than VL (40). More recently, another mechanism of diversification through V gene replacement has been reported in murine B cells but may occur only as a low frequency event (42-45). It is interesting to note, however, that a similar mechanism involving gene conversion has been described as a major means of V gene diversification in avian B cells (46-48).

Antigen-binding Domains of Immunoglobulin

The fully assembled IgG antibody molecule consists of two identical light chains and two identical heavy chains, each composed of autonomously folding domains (2 for light chains and 4 or 5 for heavy chains) of approximately 100 amino acids each. All immunoglobulin domains share an overall conserved structure consisting of two disulfide-linked β-sheets packed face to face (46). The topology of the NH2-terminal domains of the heavy and light chains (VH and VL, respectively) differs from that of the other domains by the presence of one (VH) or two (VL) additional β-strands (46,47).

Antibody specificity is determined by the sequence and size of hypervariable regions which line the combining site pocket or groove formed by the VH-VL dimer (48). These regions (see Figure 3, p.130) correspond to sequences shown by Wu and Kabat (49) to be highly variable among VH and VL genes. Thus, three hypervariable regions are donated by the heavy chain (H1, H2, and H3) and three more are donated by the light chain (L1, L2, and L3). H1 and L1 regions link β-strands in different β-sheets of their respective

domains while H2, H3, L2, and L3 are hairpin loops which link two adjacent β-strands (48).

Recent resolution of the atomic structure of several Fab and Fab-ligand complexes has greatly increased our knowledge of immunoglobulin V domain structure (47,48, 50-55). A general finding from these data is that the overall geometry of VH-VL pairs is highly conserved (47,48,49). Chothia and coworkers (47,48) reported that the root mean square differences obtained by superposition of the main chain β-framework of six known VL structures varied between 0.50 and 1.61 Å and a similar calculation for four VH domains ranged from 0.64 to 1.42 Å. These comparisons demonstrate that the framework of different immunoglobulin molecules can be very similar. Functional evidence for this comes from the fact that hypervariable regions can be transplanted from one framework to another and still retain the original specificity (56). The highly conserved β-framework extends to residues adjacent to hypervariable regions. Residues within the hypervariable regions themselves may vary up to 3 Å or more when superposed.

The observation that certain residues within the hypervariable regions are, in fact, conserved has led to the proposal that these residues influence structure rather than specificity (57). Analysis of these positions in immunoglobulins of known structure allowed Chothia and Lesk (48) to identify both hypervariable and framework residues which most likely influence the conformation of hypervariable loops through packing, hydrogen bonding or the ability to assume unusual conformations (e.g. Gly, Pro, Asn, Asp). Using this information, a successful prediction of the framework, positioning of the hypervariable regions, and antigen-contact residues of Fab D1.3 was made prior to determination of its structure in an Fab D1.3-lysozyme complex (52,58).

Analysis of available structural data has also demonstrated that residues forming the VH-VL interface are contributed by both hypervariable and framework regions (47,59,60). Twelve of the twenty interface residues are highly conserved in all known immunoglobulin sequences whereas the remaining eight positions, which are associated with the hypervariable regions, are found to be more variable (47). Novotny and coworkers (61) compared the least squares hyperboloid fit of the VH-VL interface β-barrel from the known structures of six Fv fragments and concluded that the dimensions were "virtually identical". Superposition of residues lining the VH-VL interface in the same structures also demonstrated conservation of the VH-VL interface geometry (50,61). A significant point emerging from these studies is that the overall geometry of V domains appears to be conserved in both liganded and unliganded Fabs, suggesting that conformational changes are not induced in Fabs upon antigen (hapten or protein) binding (50,52,53,62). However, Colman et al. (55) have reported a large deviation from conserved VH-VL associations in a 3-Å resolution of the x-ray structure of the Fab NC41-neuraminidase complex, favoring the interpretation that VH-VL slippage had occurred to accommodate antigen binding. Further refinement of the x-ray data and information on the atomic structure of unliganded NC41 Fab will be required in order to confirm this interpretation.

Antigen Selection and the Evolution of V Region Structure

The driving force behind acquisition of high affinity antibody and immune memory is selection by the immunizing agent (63-66). Since selection of specific B cells must involve stimulation via the immunoglobulin receptor,

somatic mutation of immunoglobulin V genes provides a diverse array of substrates on which the selection process can operate.

Somatic hypermutation appears to be triggered by a T cell dependent, antigen-induced proliferation of the B cell and is restricted to sequences of and adjacent to the rearranged V gene (41,67,74). These randomly introduced mutations occur in a stepwise manner as indicated by the observation of shared and individual mutations among clonally derived progeny (27,68-70). The rate of somatic mutation has been estimated by several groups to approach 1×10^{-3} per base pair per generation (65,69,71). As one may expect from a random process, somatic mutation introduces both selectable (beneficial or deleterious) and non-selectable (silent and flanking) changes into the V regions (72,73). The studies of Siekevitz et al.(75) and Manser and coworkers (72,76,77) have demonstrated the existence of variant clones in which loss of antigen-binding capacity is correlated with a high frequency of mutation. It has also been suggested (72,75) that the generation of variant clones may provide an opportunity for their recruitment into other ongoing antibody responses.

The distribution and nature of accumulated mutations may provide information concerning particular locations within the V region structure which can be positively or negatively selected by antigen. Mutations accumulated in regions which do not play a role in maintaining antigen-binding function appear random and the ratio of replacement (R) to silent (S) mutations, the R:S value, typically approaches 2.9 (73). The R:S value observed for antibody framework regions (73) has been 1.5 or less suggesting that conservation of the framework structure is important in maintaining antigen-binding; replacement mutations in this region may lead to total loss of the clone's participation (a "lethal" mutation). Somatic mutations

accumulating in the hypervariable regions, however, generally indicate no requirement for conservation as R:S values may be observed to approach 2.9 (40,73). In fact, the observation of R:S values much greater than 2.9 (approaching 10) is taken as significant evidence that positive selection for replacement changes occurs. The fact that hypervariable regions are associated with the antigen combining site argues favorably for the role of antigen as the selective agent. Several studies have demonstrated (27,78,79) that introduction of a single point mutation into hypervariable sequences can lead to several-fold increases in affinity for antigen. Others have shown (80,81) that single mutations can significantly alter the specificity of a given antibody combining site. Antigen selection exerted upon clones generated by the hypermutational mechanism is therefore a significant contributor to the observed changes in affinity and fine specificity associated with maturation of the antibody response.

Recruitment of newly arising B cells into an established immune response may contribute to the observed heterogeneity often observed in secondary and tertiary responses. In studies reported by Berek and coworkers (82), hybridomas resembling primary response clones of the response to 2-phenyl-oxazolone were obtained from hyperimmunized mice by altering the route of antigen administration and time of splenic fusion. It was concluded that the immune response must be capable of continuously searching among primary clones for potentially useful new germline gene combinations to recruit into the established B cell pool.

The work of Manser and coworkers suggests a strong correlation between dominance of a clonal species and its ability to produce antibodies of high affinity. The response to *p*-azophenylarsonate (Ars) in A/J mice is dominated by antibodies bearing a characteristic idiotype (IdCR) although the gene

combination encoding IdCR is not present at a higher frequency than that of another less represented anti-Ars clonotype (Id36-60) (83). Manser et al. (64,72) reason that in the presence of limiting amounts of antigen, clonal success depends not only upon intrinsic affinity for antigen, but on the ability to retain or enhance functional binding in the face of somatic mutation. Thus, IdCR-bearing antibodies are 'best fit' to respond to Ars challenge. However, even the dominance of IdCR clones in individual mice is subject to fluctuations of the randomly available V gene combinations and other environmental effects (see below). In the serum response of individual A/J mice the ratio of $IdCR^+$ to $Id36\text{-}60^+$ antibody was not found to be strictly regulated and varied over a 300-fold range(83).

The mechanisms governing antigen selection are apparently not straightforward. While the responses to some antigens display dominance by one or a few clonotypes (84-88), others demonstrate clear heterogeneity (89-91). A feature generally common to T cell-dependent secondary responses, however, is the appearance of somatically mutated antibodies of higher affinity. Finally, the observation that combining sites specific for the same or similar antigens can undergo convergent or parallel somatic evolution (65,66,92) argues strongly that antigen selection is the major influence in determining the composition of the established B cell repertoire.

Environmental Influence on the B Cell Response

The dynamic nature of lymphocyte populations and the influence of other cell types must be considered for full comprehension of the series of events resulting in acquired B cell immunity. The rate of B cell production in the bone marrow of an adult mouse has been estimated at 5×10^7 B cells per day

(93). These newly formed (primary) B cells represent approximately 20% of the existing peripheral (secondary) B lymphoid population. Under physiologic conditions, the life span of a new B cell is very short (24-72 hrs) (93,94) unless it is among the small proportion of primary B cells which undergoes suitable activation and is recruited into a pool of long-lived B cells. Thus, only a fraction of the total potential repertoire of V gene combinations is available for primary B cell activation at any given moment.

The activation process for primary B cells has been found to differ from that of secondary B cells (94). T cell-dependent activation of primary B cells may be mediated by interdigitating cells present in the extrafollicular regions of secondary lymphoid tissues and which express class II histocompatibility antigens. These regions are rich in T cells and possess B cells concentrated at the corticomedullary junction and adjacent to lymphatic sinusoids. Secondary B cells, on the other hand, may interact with antigen presented either by the interdigitating extrafollicular cells or by follicular dendritic cells in germinal centers of secondary lymphoid follicles (94,95,96). Thymus-dependent antigens are localized for long periods on the surface of these follicular dendritic cells (95). The association of antigen with follicular dendritic cells is dependent upon the presence of specific antibody and complement and appears to be in the form of an immune complex. In addition, large numbers of T cells are found associated with germinal centers (94).

B cell activation by thymus-dependent antigens appears to proceed as two distinct phases. First, activation of primary B cells in extrafollicular regions gives rise to either secondary B cells, which join the long-lived peripheral pool, or to terminally differentiated plasma cells, which secrete specific antibody at the site of activation (94). In the second phase, secondary B cells can now access antigen associated with follicular dendritic cells and, upon

activation, give rise to plasma cells or more memory B cells. Plasma cells derived from secondary B cells generally migrate to the bone marrow or lamina propria of the gut (94,97,98). The significance of a two-phase, compartmentalized activation process for the generation of memory cells may be related to the suggestion that somatic mutation is only associated with the second phase of B cell activation in germinal centers (94). This is consistent with the observation that the hypermutation mechanism is active only at certain times during B cell development (41).

T cells and their cytokines, as well as other cell types, undoubtedly play a significant role in regulating B cell activation and antibody production in general. The mechanisms involved in this regulation, however, are not yet well-defined. A role for specific T suppressor cells or T helper cells in regulating clonally restricted antibody responses through idiotypic interaction has been suggested (72,92) but unconfirmed.

The nature of the antigen itself presumably will dictate which regulatory elements are to contribute to the specific antibody response. For example, the antibody response to certain thymus-independent antigens, e. g. polysaccharide, is apparently not associated with the somatic hypermutation (99). Moreover, B cells activated by TI antigens show little or no tendency to migrate (100). Many restricted B cell responses are associated with antigenic determinants found on environmental pathogens (101). It is possible, therefore, that selectively advantageous antibody responses to such pathogens (represented in a particular V gene combination) become fixed during the evolution of species.

The Antibody Response to PC-KLH

The response to phosphocholine, an endogenous bacterial surface antigen, has served as a model system in numerous laboratories investigating the molecular basis of antibody diversity and antibody recognition. Studies in our laboratory have focused on the memory antibody response to phosphocholine coupled to keyhole limpet hemocyanin (PC-KLH) and have established that the memory response to PC-KLH consists of two major antibody populations (102,103,104): Group I antibodies, most of which bear the T15 idiotype, recognize the haptens phosphocholine (PC) and nitrophenyl phosphocholine (NPPC) equally. Group II antibodies recognize NPPC, which resembles the immunizing form of PC, much better than free PC; Group II antibodies do not bear the T15 idiotype. Group I and Group II antibodies co-dominate in the memory response to PC-KLH (91) in contrast to the primary response in which Group I antibodies comprise at least 95% (105-107) of the antibody. Thus, Group II antibodies undergo selective expansion from a minor population in the primary response to be equally represented in the memory anti-PC-KLH response. The mechanism of this expansion to co-dominance despite overwhelming Group I dominance of the primary response is unclear and cannot be simply explained on the basis of affinity for the hapten NPPC. Fluorescence quenching experiments have determined that Group I and Group II monoclonal antibodies have comparable affinity for NPPC (Bruderer et al., unpublished results) consistent with the finding that early phase Group I and Group II serum antibodies (day 17 of a primary response) are inhibited from binding to PC-protein in an ELISA by comparable amounts of NPPC (91). Interestingly, day 17 Group II antibodies were found to be 10^3 - 10^4 fold more sensitive to inhibition by PC-protein conjugates than Group I antibodies (91).

Thus, it is possible that Group II antibodies can compete effectively in this response by initially having much greater affinity for the immunizing form of PC (i.e. coupled to KLH via a diazophenyl linker). This suggests that one factor playing a role in memory development to PC-KLH is the differing recognition of the PC-phenyl-protein epitope by Group I and Group II antibodies.

The dominance of Group I antibodies in the response to PC-bearing bacteria and in the early PC-KLH response is well-documented (105-107). It has been proposed that this restriction results from a selective pressure to evolve an optimal antibody response, represented by the T15-positive Group I response, which has been shown to be protective against PC-bearing bacteria (101,108). It is possible that the initial Group I dominance is lost in response to PC-KLH as a result of new Group II B cell clones arising with much greater avidity for the protein coupled form of PC rather than the bacterial form of PC. Preliminary experiments using analogs of NPPC in competition assays in order to identify structural regions comprising the epitope recognized in Group I vs Group II antibodies suggest that Group I and Group II antibodies do not recognize the PC-protein epitope in an identical manner (91,109). It is possible that structural recognition and affinity govern, at least in part, the rapid expansion of Group II antibodies. The phenyl linker appears to play a dominant role in this recognition and may play such a dominant role in responses to phenyl-containing structures in general.

The evolution of V gene usage in the development of immune memory to PC-KLH and its correlation with structural recognition of the epitope lends another significant aspect of immune maturation. V gene usage in Group I antibodies is highly restricted to use of the VH1 (S107 family) gene in combination with the Vk22 light chain gene and, less frequently, with the

Vk24 or Vk8 genes (110). Concomitant with the shift to Group II fine specificity phenotype is the appearance of B cell clones utilizing novel VH and VL gene combinations (91,109,111). At least 5 different VH gene families can participate in the Group II response (91,109,112) indicating that heterogeneity exists in both VH gene usage and fine specificity. However, 2 VL families not associated with Group I antibodies, Vk1 and Vλ, participate prominently in the Group II memory response (91,109). The Vk1 family has been suggested to play an important role in encoding antibodies which recognize phenyl-containing structures (19,113). The finding of relatively diverse VH use in combination with more restricted VL use suggests that light chains may be a more important determining factor of specificity in the anti-PC-KLH response.

Structural Features of a PC-specific Combining Site

The structure of the McPC603 (M603) combining site is highly relevant to the work presented here as its Fab structure complexed with the organophosphorus hapten phosphocholine (PC) has been determined (51,54). The antigen-contact residues of M603 have also been found conserved in several other PC-binding myeloma proteins (79-114). The binding site of M603 forms a wedge-shaped cavity, approximately 15 A x 20 A in length and 12 A in depth. The hapten PC is asymmetrically bound and makes more contact with heavy chain residues than with light chain residues. Only five hypervariable regions (L1, L3, H1, H2, H3) were found to contribute to antigen binding in M603; the L2 region is blocked from approaching the hapten binding site by the large loops of L1 and H3. Additional residues present in the L1, H2, and H3 regions cause an extension of these loops and add to the depth of the binding cavity.

The orientation of bound PC is such that the positively charged choline group is buried in the combining site and the phosphate group is positioned closer to the mouth of the cavity. Such an orientation seems logical since PC is associated with capsular polysaccharides of pneumococci, for example, by an ester linkage involving the phosphate group (114). An electrostatic interaction involving Arg 52H and the negatively charged phosphate of PC contributes significantly to the binding energy of the hapten-antibody complex (54,61). Contact of the phosphate is also made through hydrogen bonding of the Tyr 33H hydroxyl. Residues interacting with the positively charged nitrogen of the choline group include side chains of Glu 35H and Glu 58H and the main chain atoms of light chain residues 91-94 (Asp 91, Ser 92, His 93, Tyr 94). Extensive contact of the hapten is made by the side chains of Trp 103H, Tyr 33L and Leu 96L.

The M603 combining site has been utilized as a structural reference for the modelling of several homologous proteins (48,79,114). L1 residues 26 to 27b in the light chain of M603 extend across the top of VL with the side chain of Leu 27b buried within the framework. L1 residues 27c to 31 form a poorly ordered hairpin loop extending away from the general surface. L2 residues 50-52 participate in a three-residue hair-pin turn in which all side chains are oriented towards the surface. The L3 region of M603 includes Pro95 which is present in a cis conformation and promotes an extension of residues 93-96 rather than a hairpin loop. Furthermore, the amino side chain of Asn 90 forms hydrogen bonds with the main chain atoms of Ser 93 and Pro 95. The hypervariable loops in the M603 heavy chain follow a layout similar to that of the light chain. H1 residues 26-32 extend across the VH framework with Phe 29 buried, a sharp turn being produced by the presence of Gly 26H. H2 residues 52-56 form part of a ten-residue hairpin turn involving part of the

conserved framework. The H3 region of M603 includes a nine-residue hairpin turn determined by a salt bridge formed between Arg 94 and Asp 101, and the participation of Tyr 100b and Tyr 100c as interface residues. Disruption of this salt bridge by a mutation to Ala 101 in a related protein encoded by the same VH as M603 resulted in complete loss of PC binding (72).

The Antibody Response to Soman-KLH

Soman (0-1,2,2,trimethylpropylmethylphosphonofluoridate) is an organophosphorus neurotoxin which acts as a potent and longterm inhibitor of cholinesterases causing respiratory paralysis (115). Recent interest in the generation of antibody specific for Soman has been precipitated by the unfortunate production and stockpiling of Soman as a potential chemical warfare agent (115,116). Lenz and coworkers (117.118) investigated the ability of two monoclonal antibodies generated against a Soman-phenyl-protein conjugate to protect or act therapeutically against Soman intoxication and obtained limited success. Their observation that the specificity of these anti-Soman monoclonals and of polyclonal rabbit serum antibody against Soman conjugates included recognition of the phenyl linkage to the protein carrier is consistent with the findings reported here.

Our interest in the immune response to Soman is its potential use as a model system in elucidating mechanisms which regulate the generation and selection of specific antibody responses. The study of specific antibody formation in response to defined haptenic structures continues to be highly informative regarding the relationship of antibody structure to antigen recognition.

The haptens Soman and PC appear to be similar in spatial orientation, particularly when coupled to protein carriers via a diazophenyl linkage (See Table I, p. 52). The two most notable differences between Soman and phosphocholine involve charge changes in which 1) the positively charged nitrogen in the choline moiety of PC is replaced with an uncharged carbon in Soman and 2) the negatively charged oxygen of the phosphate group in PC is instead a methyl group in Soman. In addition the Soman molecule is one carbon length shorter than PC between the phosphonate and the free end and instead has a branched methyl group at carbon-1. The Soman-KLH response serves as an appropriate model system to complement investigation of immune memory development to phenyl-bearing structures such as PC-KLH since by lacking the charged groups present in PC, comparable V gene usage or structures found in the Soman response would emphasize the contribution of the phenyl moiety. We have therefore investigated the murine antibody response to Soman-KLH at the fine specificity and molecular levels and present here our results relative to what is known of other antigen-specific responses, emphasizing its relationship to the anti-PC-KLH response in particular.

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Paper 1

Combining Site Specificity of Monoclonal Antibodies to the Organophosphorus Hapten Soman

Abigail C. Buenafe and Marvin B. Rittenberg

ABSTRACT

The combining site specificities of eight monoclonal antibodies raised against the organophosphorus-containing hapten Soman are compared to monoclonal antibodies specific for a naturally occurring organophosphorous compound, phosphocholine (PC). Although these haptens share some structural and spatial features, differences in their chemical structures, most notably the presence or absence of a positive charge, appear to prevent significant cross-reactivity between antibodies specific for each.

The murine memory response to PC-KLH has been shown previously to be characterized by the presence of two major groups of antibodies differentiated on the basis of their specificity for free PC and for the nitrophenyl derivative of PC, nitrophenylphosphocholine (NPPC). Interestingly, we have detected two groups of hybridoma antibodies in the immune response to Soman-KLH which possess differential specificity for Soman and for a nitrophenyl derivative of Soman.

INTRODUCTION

Soman (0-1,2,2,trimethylpropylmethylphosphonofluoridate) is an organophosphonate which paralyzes the nervous system by binding to the active site of the enzyme acetylcholinesterase and inactivating the enzyme through phosphorylation of the active serine residue (1). We have prepared murine monoclonal antibodies against Soman in order to assess the variety of ways in which antibodies may recognize this structure. In addition, we have compared the specificities of these antibodies with other monoclonals against a naturally occurring organophosphorus-containing hapten, phosphocholine (PC). PC is a common environmental antigen found in the membranes of many bacteria, fungi, and mammalian cells. It has been used frequently as an antigen and the immune response to PC is well-characterized. The memory response to PC-KLH is characterized by the presence of two main groups of antibodies which have been described in detail (2). In terms of fine specificity, Group I antibodies bind to PC-protein and the binding is inhibited by both free PC hapten and by its hapten analog nitrophenyl-PC (NPPC). Group II antibodies also bind specifically to PC-protein but binding of this group is inhibited to a greater extent by NPPC than by free PC (2,3).

Soman shares limited spatial and structural properties with PC and both may be coupled to protein carriers via a diazophenyl linkage in an identical manner. In this paper the fine specificity of monoclonal antibodies representative of the anti-Soman response is compared to that of monoclonal antibodies representing Group I and Group II of the anti-PC response. In addition we show that despite the similarities between the general structures of Soman and PC and the types of antibody specificities they elicit, there is

very little cross-reactivity of the antibodies elicited in these two systems due, at least in part, to a charge difference between these two haptens.

MATERIALS AND METHODS

Animals:

Female BALB/c mice, 4 to 6 weeks of age, were obtained from Jackson Laboratory, Bar Harbor, Me. They were first immunized at approximately 10 to 12 weeks of age.

Haptenation of protein carriers:

Soman-KLH and Soman-BSA were prepared according to a modified method of Chesebro and Metzger (4). Briefly, aminophenyl-Soman (a gift from Dr. D. Lenz, USAMRICD, Aberdeen Proving Ground, Md.) was diazotized with nitrous acid and reacted with keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) in borate-saline buffer, pH 9.2, for two hours at room temperature. Soman-KLH was adjusted to pH 8 and dialyzed against borate-saline buffer, pH 8.0. Soman-BSA was adjusted to pH 7 and dialyzed against phosphate-buffered saline, pH 7.0. The hapten to protein molar ratio was determined (4) to be So_{1356}-KLH and So_{26}-BSA using a molecular weight of 8×10^6 for KLH and 7×10^4 for BSA (5). PC-histone and PC_{11}-BSA were prepared as described previously (3,5).

Immunizations:

Mice were immunized against Soman by three injections of So-KLH (100 μg) given intraperitoneally at two week intervals; the first injection was given in Complete Freund's Adjuvant, the second and third in Incomplete Freund's Adjuvant. After a two month rest, mice were boosted intraperitoneally with 100 μg So-KLH/CFA followed 2 weeks later by 50 μg So-KLH in saline intravenously.

Hybridomas:

Spleen cells were removed four days after the last injection of So-KLH and the cells fused with the non-secreting myeloma line FO according to the method of Fazekas de St. Groth and Scheidegger (6). The fused cells were cultured in 96-well microculture plates (Falcon, Oxnard, CA) at a density of 5 x 10^5 cells per well and grown in the presence of HAT-selective medium. Supernatants were screened for Soman-specific antibody by ELISA using polystyrene plates (Costar, Cambridge, MA) coated with 10 µg/ml So-BSA. The plates were blocked with PBS-1% BSA, incubated with the supernatants, and then incubated with alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin. Positive wells were detected by addition of the substrate p-nitrophenylphosphate (NPP, Sigma Chem. Co., St. Louis, MO). Absorbance was read at 410 nm with an automatic microplate reader (Model MR600, Dynatech, Alexandria, VA). Positive hybridomas were cloned twice by the single cell hanging drop technique (7). Isotypes of the eight resulting monoclonals were determined in an ELISA using class- and subclass-specific reagents (3) (Zymed, San Francisco, CA). Soman-specific antibodies were raised as ascites (4), then purified by affinity chromatography on Soman-glycyltyrosyl-Sepharose 4B and eluted with 3 M NaSCN, pH 8.0. The production of PC-specific hybridomas and the subsequent characterization of Group I and Group II anti-PC monoclonal antibodies have been described previously (8). PCG1-4, which was derived from a primary response fusion, and PCG3-1, derived from a memory response fusion, are two Group I monoclonals which have not been described previously.

Fine specificity analysis of hapten-specific antibodies:

The fine specificities of Soman-specific monoclonal antibodies were determined in an ELISA inhibition assay. Anti-Soman antibodies were incubated on plates coated with 10 µg/ml So-BSA in the presence of varying concentrations of inhibitor. The inhibitors included the following Soman derivatives: dipinacolylmethylphosphonate (DPMP), diisopropylmethylphosphonate (DIMP), p-aminophenyl-Soman (APSo), p-nitrophenyl-Soman (NPSo), and hydroxy-Soman (OH-So) (Dr. D. Lenz, USAMRICD, Aberdeen Proving Ground, Md.). Table 1 illustrates the chemical structure of these compounds. Other inhibitors used were PC and NPPC (Sigma Chem. Co., St. Louis, MO), PC_{11}-BSA, So_{26}-BSA, and So_{1356}-KLH. An additional Soman derivative, 1-amino-methoxy-Soman (kindly provided by Dr. R.M. Moriarty, University of Chicago, Chicago, Ill., Table 1) was used as a competitive inhibitor in the binding of anti-Soman and anti-PC antibodies to Soman-BSA and PC-histone-coated plates, respectively. After incubation, the plates were washed and incubated with alkaline phosphatase-labeled isotype-specific reagents (3) and the color reaction developed by addition of NPP. The percent inhibition was calculated by quantitation of the antibody bound in the presence of varying amounts of inhibitor using a standard curve set up with each assay. The I_{50} value for each inhibitor was calculated according to the method of Reed and Muench (9) and is expressed as the millimolar concentration required for 50% inhibition of anti-Soman antibody binding to So-BSA.

RESULTS

Fine specificity analysis of Soman-specific monoclonal antibodies:

The fine specificity of eight monoclonal anti-Soman antibodies was determined by an ELISA inhibition assay in which various derivatives of Soman were used as inhibitors. Table 2 provides a summary of this analysis by giving the I_{50} value obtained for each inhibitor. Four of the eight Soman antibodies, So-G3-1, So-G3-2, So-G3-3, and So-G2a-1 are able to bind the hapten DPMP, which does not carry a charge and which lacks a phenyl ring, as well as the aminophenyl and nitrophenyl derivatives of Soman as determined by the ability of these haptens to inhibit the binding of anti-Soman antibodies to So-BSA-coated plates. So-G3-1, So-G3-2, So-G3-3, and So-G2a-1 have been designated as Group A anti-Soman antibodies. Hapten inhibition curves for So-G3-2 as representative of Group A anti-Soman antibodies are shown in Fig. 1A using DPMP and NPSo as inhibitors.

Three other antibodies, So-G1-1, So-G2a-2, and So-G2b-1 possess much greater affinity for aminophenyl- and nitrophenyl-Soman than for DPMP, for which binding was not demonstrable. These antibodies have been designated as Group B antibodies. It is possible that the combining sites of these antibodies are directed primarily at the phenylphosphate region of the molecules which is present in the immunizing form of Soman. For example, we have found that So-G1-1 can be inhibited by high concentrations of p-nitrophenylphosphate (I_{50} = 5.8 mM) suggesting that the phenylphosphate region can be recognized by So-G1-1. However, the specificity of this antibody is not restricted to the phenylphosphate structure since the I_{50} for NPSo is 10,000 fold lower (0.0004 mM, Table 2). It is probable that these antibodies also recognize the remaining portion of the Soman molecule in addition to the

phenylphosphate region since some Group B monoclonals, including So-G1-1, also appear to be inhibited by very high concentrations of hydroxy-Soman which does not contain the phenylphosphate structure. Inhibition curves are shown for So-G1-1 in Fig. 1B using DPMP and NPSo as inhibitors and are representative of all Group B proteins listed in Table 2. It is clear that the inhibition patterns for Group A antibodies and Group B antibodies (Fig. 1A vs. 1B) are distinct.

So-M-1 was not inhibitable by any of the haptens tested and thus could not be assigned to either Group A or Group B. As shown in Fig. 2, the binding of So-M-1 to So-BSA-coated plates is inhibited by So-BSA or So-KLH but not by PC-BSA, BSA or KLH thus indicating that it is indeed specific for Soman. We have characterized ten other hybridomas with similar fine specificity (not shown) and, interestingly, all are IgM proteins although at least one uses λ rather than κ light chains. It may be that So-M-1 possesses a very low affinity for Soman and is, therefore, much more difficult to inhibit by free hapten. The avidity of the interaction, i. e. the binding of a pentameric structure versus a monomeric structure to an antigen-coated surface, may also contribute to the difficulty in obtaining inhibition by hapten.

Finally, the binding specificities of anti-Soman Group A and Group B antibodies were compared to those representative of anti-PC antibodies. PCG3-1 and PCG2b-2 (10) were used as examples of anti-PC Group I and Group II antibodies, respectively. Fig. 3(A,B) demonstrates the differential affinities of PCG3-1 and PCG2b-2 for the haptens NPPC and PC (2,10,11). The similarities between anti-PC and anti-Soman antibodies demonstrated with the specificity profiles for haptens possessing and lacking the phenyl ring are striking when Fig. 3 is compared with Fig. 1 and is consistent with the suggestion that

two distinct antibody phenotypes are represented in each case as discussed below.

Non-crossreactivity of anti-Soman and anti-PC antibodies:

It was of interest to determine the degree of cross-reactivity between antibodies specific for the haptens Soman and PC. To do this, we tested for the direct binding of PC-specific antibodies to Soman-BSA- or Soman-KLH-coated plates in an ELISA. No detectable anti-PC antibody was bound to the plates. Likewise, anti-Soman monoclonal antibodies did not bind to PC-histone coated plates (data not shown).

In order to look for possible cross-reactivity more closely, hapten inhibition studies were performed. In this assay, anti-PC antibodies were tested for binding to PC-histone coated plates in the presence or absence of various Soman inhibitors. Soman derivatives, including DPMP, DIMP, NPSo, and Soman-BSA, were unable to inhibit the binding of anti-PC antibodies to PC-histone in an ELISA inhibition assay as shown for PCG3-1 and PCG2b-2 in Fig. 4(A,B). Inhibition by PC-BSA serves as a positive control for the hapten specificity of the hybridoma proteins. As has been noted previously the concentration of PC hapten which is inhibitory is one or more orders of magnitude lower when it is present as a protein conjugate than when it is present as the free hapten (5). The ability of PC, NPPC, and PC-BSA to inhibit the binding of Soman monoclonals to So-BSA was also tested. No inhibition was seen except in the case of So-G1-1 which appears to bind NPPC weakly (I_{50} = 4.7 mM, Fig. 5(A,B)) as discussed below.

In addition, 1-amino-methoxy-Soman (Table 1) was used as a competitive inhibitor in the binding of anti-PC antibodies to PC-histone. A major

difference between this derivative and the other Soman analogs is the addition of an amino group to the pinacolyl moiety of Soman. Thus, this hapten carries a positive charge at neutral pH rather than having an uncharged hydrophobic end group. We reasoned that the presence of this positive charge on a Soman analog might allow it to mimic the ability of positively-charged PC haptens to be bound by anti-PC antibodies. Two Group I anti-PC antibodies, PCG1-4 and PCG3-1 (Fig. 6A,B), were inhibited by 1-amino-methoxy-Soman (I_{50}'s = 1.1 mM and 1.0 mM, respectively); in contrast the Group II anti-PC antibody, PCG1-1, was not inhibited (Fig. 6C) indicating that inhibition by 1-amino-methoxy-Soman was the result of specific interactions with PCG1-4 and PCG3-1. In addition, the prototype anti-PC myeloma protein TEPC 15, which has Group I binding specificity (2) was also inhibited by 1-amino-methoxy Soman (I_{50} = 1.0 mM) but was not detectably inhibited by the hydrophobic Soman analogs or Soman-protein conjugates (not shown). In contrast only 2/13 anti-Soman hybridoma antibodies have been inhibitable by the amino-methoxy derivative (not shown) suggesting that for most anti-Soman antibodies a positive charge on the pinacolyl moiety negatively influences binding.

DISCUSSION

Monoclonal antibodies raised against Soman and PC are essentially non-cross-reactive and are, therefore, highly specific for their respective haptens. The use of a common diazophenylphosphate linkage to couple both Soman and PC to protein and a general similarity in spatial orientation and structure are not sufficient to produce cross-reactivity between the two types of antibodies. Distinct differences are present in the chemical structures of Soman and PC, the most notable difference being that Soman has an uncharged pinacolyl moiety in place of the positively charged choline in PC. Thus, hydrophobic interactions may be important in binding to Soman and the antibody combining sites presumably reflect this difference. The positively charged choline group as well as the negatively charged phosphate group have been shown to play key roles in the binding of PC to the active site of the anti-PC myeloma protein McPc603 (12). In fact, we show (Fig. 6) that some anti-PC antibodies which do not bind to the hydrophobic Soman analogs do bind to 1-amino-methoxy Soman (Table 1) which, at pH 7, carries a positive charge on the pinacolyl residue. The finding that two Group I anti-PC antibodies bound to 1-amino-methoxy Soman but that a Group II anti-PC antibody did not may indicate a difference in the specificity of Group I and Group II antibodies for different regions of the PC moiety.

Although no cross-reactivity between the two types of antibodies could be demonstrated, there are interesting similarities in the anti-Soman and anti-PC systems. Fine specificity analysis of the panel of Soman-specific monoclonals indicates that, as with PC-specific antibodies, they recognize at least two different regions of their cognate haptens. Group A anti-Soman antibodies appear to be directed against the unlinked end of the hapten, i. e. the pinacolyl moiety of Soman; analogously Group I anti-PC antibodies primarily recognize

the choline moiety of PC. These observations are based on their ability to be inhibited by the free haptens DPMP and PC, respectively. Similarly, Group B anti-Soman antibodies and Group II anti-PC antibodies are analogous in that they appear to have greater affinity for the phenylphosphate derivatives of their respective haptens. The observation that Group B anti-Soman and Group II anti-PC antibodies do not cross-react indicates that the phenylphosphate region alone does not determine the specificity of these antibodies. Consistent with this interpretation is the finding that nitrophenylphosphate is not a good inhibitor of antibodies of either specificity. The one exception to this is the monoclonal So-G1-1 which binds weakly to NPP indicating that the phenylphosphate region may be of greater importance in the structure recognized by this antibody. Moreover, So-G1-1 is the one anti-Soman antibody which has measurable affinity for the PC analog NPPC. In all we have found just 3 out of 47 anti-Soman hybridomas which bind NPPC (6%). Thus, So-G1-1 may represent a minor population of antibodies with specificity for structures common to both haptens.

A number of hapten systems are currently being used to characterize various aspects of the immune response. Restricted heterogeneity in the antibody response has been described at the levels of idiotypic as well as molecular genetic analysis for haptens such as PC (13-15), azophenyl arsonate (16-18), and in the primary response to 2-phenyl-5-oxazalone (19). In contrast, antibody responses to haptens such as fluorescein (20) as well as phthalate (21) appear to be extremely heterogeneous. It will be of interest to determine the degree of heterogeneity which exists in the anti-Soman response relative to the comparable but non-cross-reactive PC moiety. From 47 anti-Soman hybridoma clones we have isolated it appears that the secondary anti-Soman response is comprised of a finite (approximately 10)

number of fine specificity phenotypes, two of which as defined here parallel those of the phosphocholine response. We have yet to determine whether these variations in antigen binding result from multiple gene usage, extensive somatic mutation, or both.

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Table 1: CHEMICAL STRUCTURE OF SOMAN AND PC DERIVATIVES

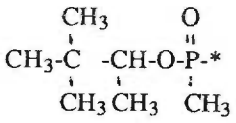
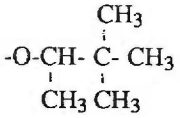
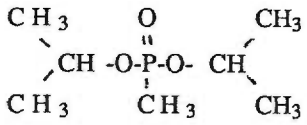
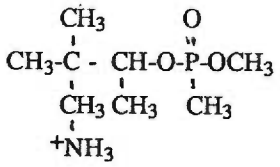
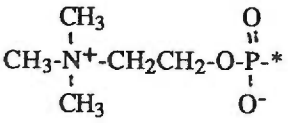
Soman Haptens:	General Formula:	
pinacolylmethylphosphonofluoridate (Soman)	*=	-F
dipinacolylmethylphosphonate (DPMP)		
p-aminophenyl-Soman (APSo)		-O-ϕ-NH_2
p-nitrophenyl-Soman (NPSo)		-O-ϕ-NO_2
hydroxy-Soman (OH-So)		-OH
Soman-protein conjugate (So-BSA, So-KLH)		-O-ϕ-N=N-protein
diisopropylmethylphosphonate (DIMP)		
1-amino-methoxy-Soman		
PC Haptens:	General Formula:	
phosphocholine (PC)	*=	-OH
p-nitrophenylphosphocholine (NPPC)		-O-ϕ-NO_2
PC-protein conjugate (PC-BSA, PC-Histone)		-O-ϕ-N=N-protein

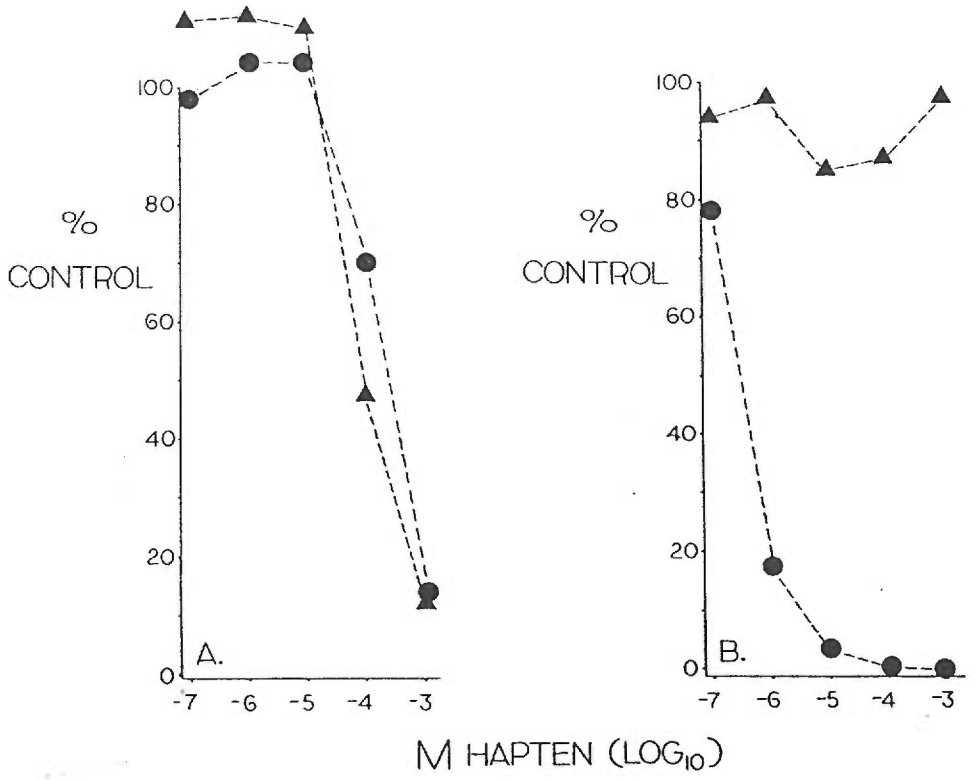
Table II: I_{50} values (mM) for Soman-specific hybridoma antibodies

	INHIBITORS:					ISOTYPE:
	DPMP	NPSo	APSo	OH-So	DIMP	
Group A						
So-G3-1	0.315	0.127	0.062	>10	>10	IgG3
So-G3-2	0.090	0.234	0.059	>10	>10	IgG3
So-G3-3	0.092	0.275	0.060	>10	>10	IgG3
So-G2a-1	1	0.071	0.033	>10	>10	IgG2a
Group B						
So-G1-1	>1	0.0004	0.002	2.49	>10	IgG1
So-G2a-2	>1	0.0008	0.018	*7.27	>10	IgG2a
So-G2b-1	>1	0.035	0.022	>10	>10	IgG2b
So-M-1	>1	>10	>1	>1	>10	IgM

(*value is variable between 7.26 and >10)

Fig. 1: Inhibition of anti-Soman hybridoma antibodies by Soman analogs DPMP (▲) and NPSo (●).

(A) So-G3-2, Group A; (B) So-G1-1, Group B.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-4
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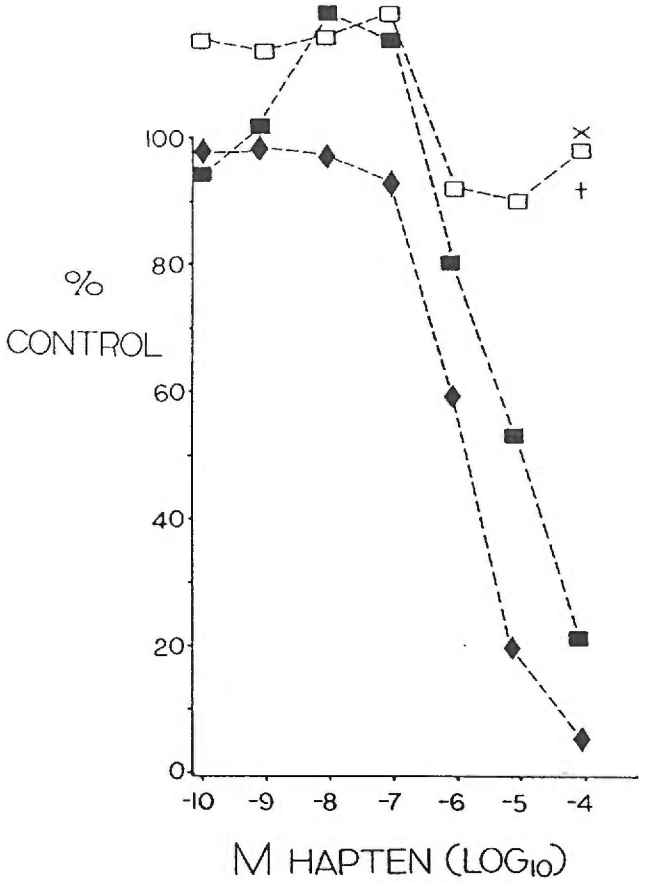
M HAPTEN (LOG10)

Fig. 2: Inhibition of So-M-1 by So_{1356}-KLH (♦), So_{26}-BSA (■), PC_{11}-BSA (□), KLH (2 mg/ml,+), BSA (10 mg/ml, x).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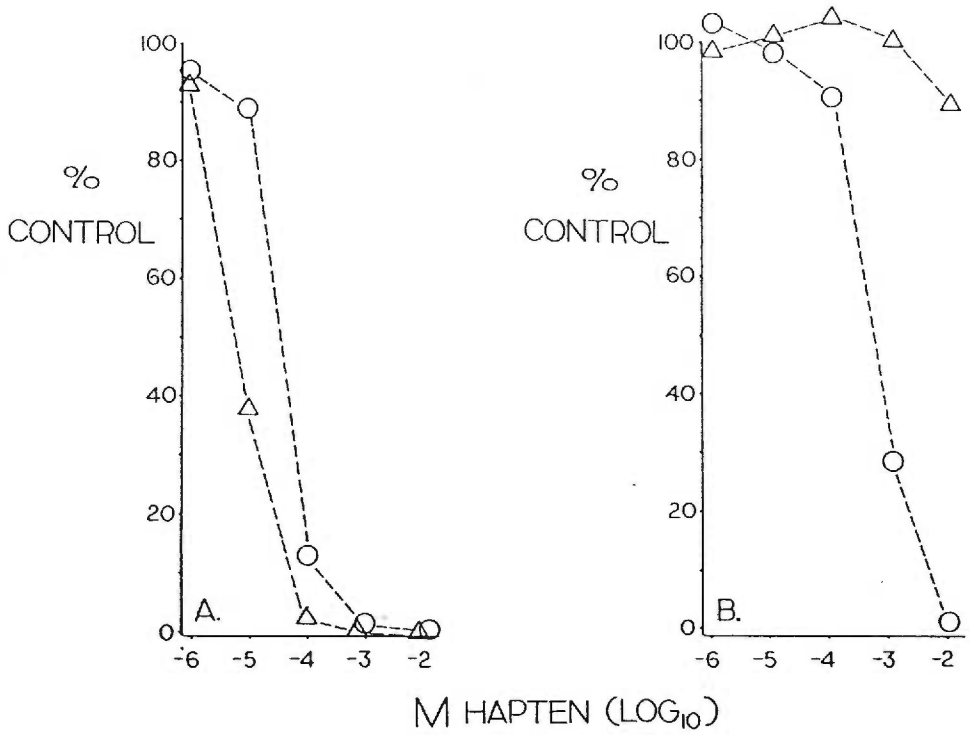
-5
-4
M HAPTEN (LOG10)
X
+

Fig. 3: Inhibition profile of anti-PC hybridoma antibodies by PC (△) and NPPC (o). (A) PCG3-1, Group I; (B) PCG2b-2, Group II.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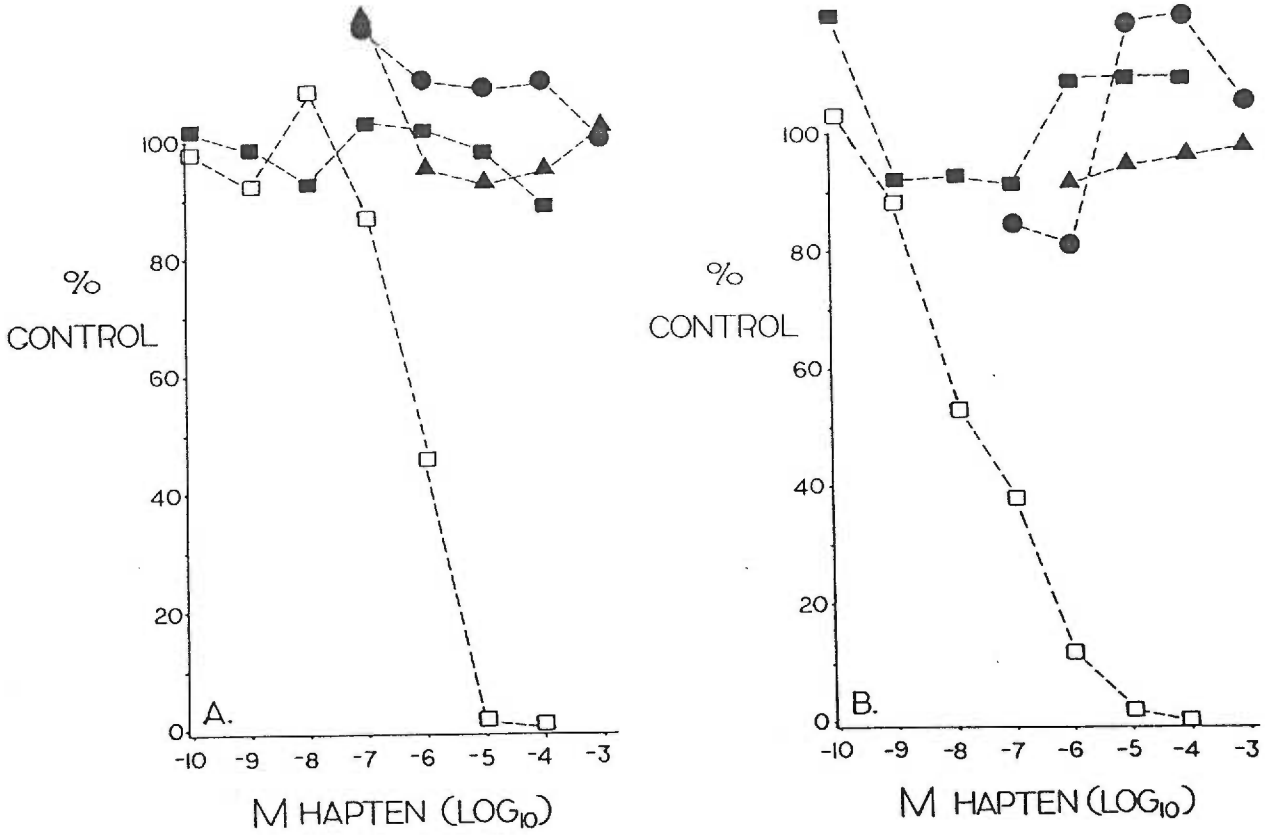
B.
M HAPTEN (LOG10)

Fig. 4: Anti-PC hybridoma antibodies are not inhibited by Soman haptens: DPMP (▲), NPSo (●), So-BSA(■). PC-BSA (□) serves as a positive control. (A) PCG3-1; (B) PCG2b-2.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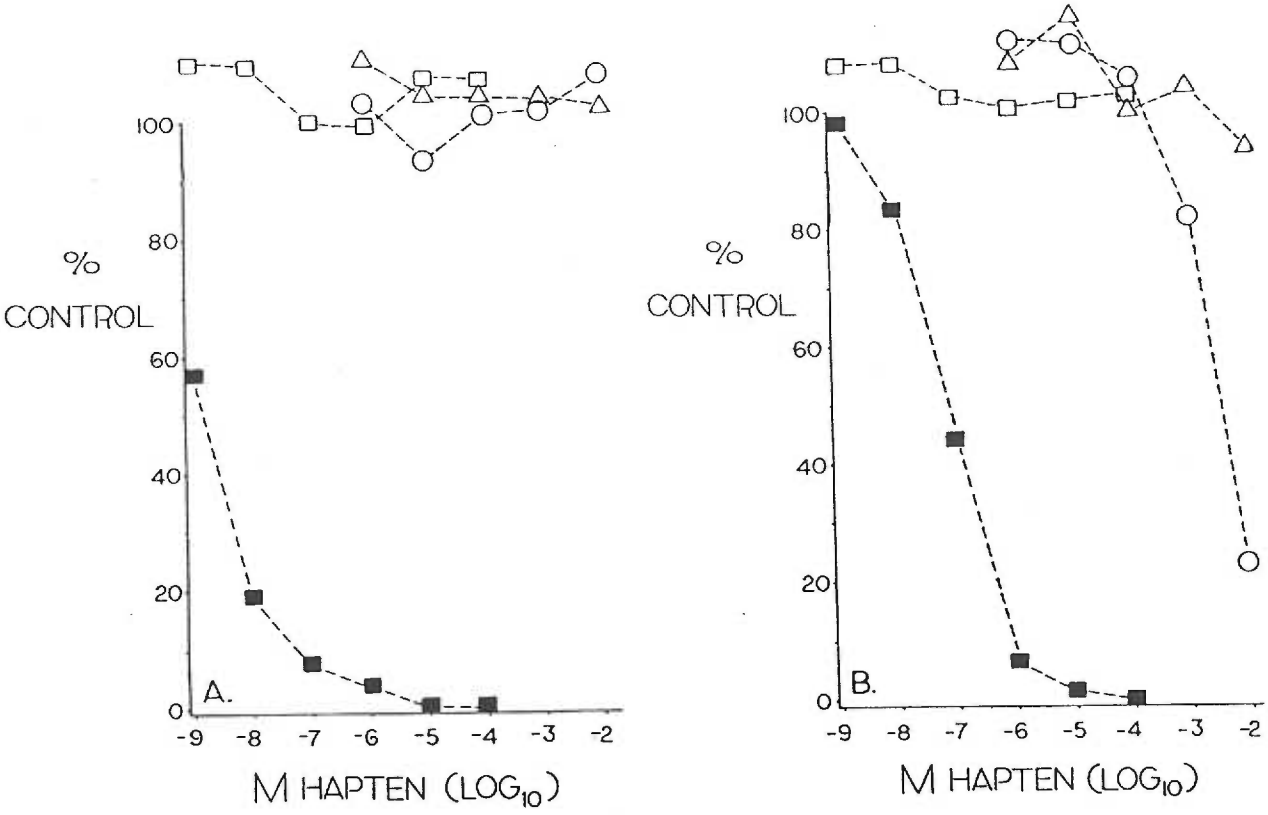
M HAPTEN (LOG10)

Fig. 5: Anti-Soman hybridoma antibodies are not generally inhibited by PC haptens: PC (△), NPPC (o), and PC-BSA (□). So-BSA (■) serves as a positive control. (A) So-G3-2; (B) So-G1-1 is inhibited by high concentrations of NPPC only (see text).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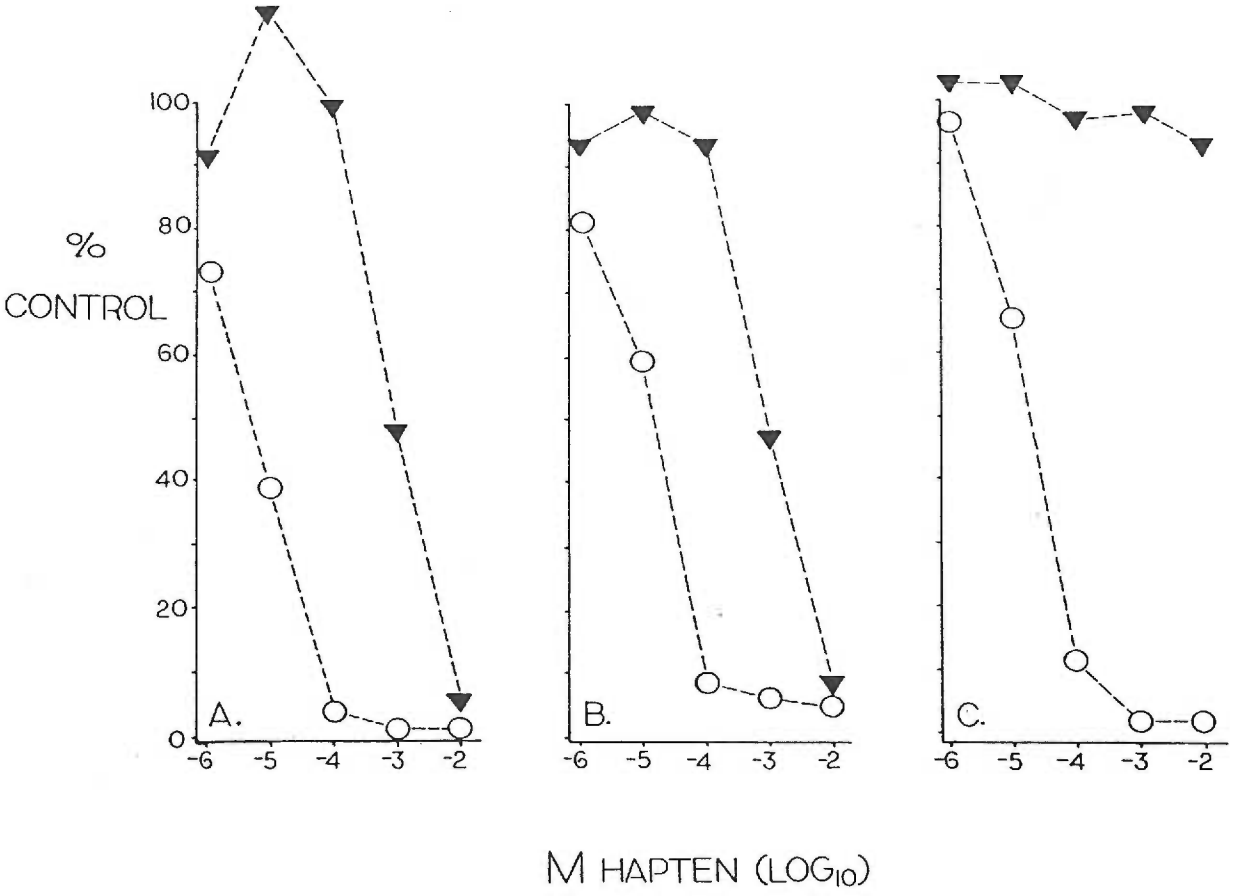
M HAPTEN (LOG10)

Fig. 6: Inhibition of anti-PC antibodies by 1-amino-methoxy-Soman (▼); (A) PCG1-4, Group I; (B) PCG3-1, Group I; (C) PCG1-1, Group II. Inhibition by NPPC (o) serves as a positive control.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M HAPTEN (LOG10)

Paper 2

Molecular Analysis and Fine Specificity of Antibodies Against an Organophosphorus Hapten

Abigail C. Buenafe, Frances F. Makowski and Marvin B. Rittenberg

ABSTRACT

We have identified four fine specificity groups reactive with the organophosphorus hapten Soman among 46 hybridomas generated in specific response to immunization with Soman-KLH. The different fine specificity groups do not appear to correlate with the use of particular V genes. Molecular analysis of VH genes demonstrates predominant use of VH J558 family members in hybridomas of all fine specificity groups although several different VH genes within this family as well as others are able to contribute. Diversity of VH gene usage was also apparent in primary IgM-producing hybridomas. In contrast, there appears to be restricted light chain usage; a large number (18/46) used the Vκ1 family. Interestingly, the Vκ1 family also plays an important role in the memory response to phosphocholine-KLH, a related organophosphate hapten which shares several structural features with Soman, particularly when coupled to protein carriers. The Vκ1C gene appears to predominate in the PC-KLH response. Restriction analysis of DNA from the Vκ1-positive Soman-KLH-specific hybridomas suggests that a single Vκ1 gene may be utilized by 17/18 but that this gene is different from Vκ1C and may be Vκ1A. We propose that members of the Vκ1 family contribute favorably in generating combining sites that recognize all or part of the structural features shared by the two haptenic structures Soman and PC when they are coupled to protein. This most likely involves recognition of the phenyl linker moiety as the dominant feature. It appears that the light chain rather than the heavy chain may play a more significant role in forming the phenyl-Soman-specific combining site and perhaps the combining sites for phenyl or ring structures in general.

INTRODUCTION

Investigations of the immune response to defined haptenic structures continue to contribute significantly to our understanding of antibody specificity and diversity (1-4). We are investigating the immune response to the hapten, Soman (0-1,2,2 trimethylpropylmethylphosphonofluoridate) for fine specificity and V gene usage. In the fluoro form Soman (see Figure 1, inset) is a potent phosphorus-containing neurotoxin (5). Our interest in the immune response to Soman-KLH stemmed from our studies of the memory response to another organophosphorus-containing hapten, phosphocholine (PC). Soman and PC appear to be similar in spatial orientation, particularly when coupled to protein carriers via a diazophenyl linkage (Figure 1). Both haptenic forms possess a distal quaternary terminal structure attached via a phosphate group to the phenyl linker. This quaternary structure is the free end in the hapten protein conjugates. The two most notable differences between Soman and PC involve charge changes in which 1) the positively charged nitrogen in the choline moiety of PC is replaced with an uncharged carbon in Soman and 2) the negatively charged oxygen of the phosphate group in PC is, instead, a methyl group in Soman. In addition the Soman molecule is one carbon shorter than PC between the phosphonate and the free end and instead has a branched methyl group at carbon 1.

Here we report the analysis of 46 BALB/c hybridomas derived after primary and secondary immunization with Soman-keyhole limpet hemocyanin (So-KLH). Four main fine specificity groups are identified by inhibition studies using hapten analogs of Soman. Three of the fine specificity groups have been described in a previous study characterizing eight of these hybridomas (6). We are interested in the molecular analysis of

the V genes utilized by these anti-Soman hybridomas for two reasons. First, we wish to know if anti-Soman antibodies of a particular fine specificity phenotype are restricted to the use of certain V genes or V gene families. Secondly the structural features presented by both the phenyl-Soman and phenyl-PC haptens led us to hypothesize that antibodies generated to these two forms might cross-react and/or be encoded by similar V genes. However, in a previous report we demonstrated that monoclonal antibodies (mAbs) specific for Soman or PC showed minimal cross-reactivity, most likely because of charge differences in the haptens (6). Since the phenyl moiety common to these haptens appears to be an immunodominant feature of the epitope, we have undertaken a comparison of V gene usage despite the lack of cross-reactivity to ask if certain V gene products are recruited specifically in response to structurally related antigens.

In this report we demonstrate that both primary and secondary antibody responses to Soman-KLH are relatively diverse with regard to VH gene usage and that fine specificity in this system does not correlate with any single VH gene or VH gene family. However, we find that one light chain gene family, Vκ1, contributes significantly; this is particularly interesting since Vκ1 also participates in the response to PC-KLH (7-9) and to a number of other aromatic or ring-bearing structures including phenyloxazolone (10), TGAL $(Tyr,Glu)_n$-$(Ala)_n$-$(Lys)_n$ (11), GAT $(Glu^{60}\ Ala^{30}\ Tyr^{10})_n$ (12) and DNA (13).

MATERIALS and METHODS

Animals:

Female BALB/c mice, age 4 to 6 weeks, were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were first immunized at approximately 10 to 12 weeks of age.

Hybridoma Production:

Three separate groups of mice were used to generate Soman-specific hybridomas as described previously (6). All mice were immunized with So-KLH and their spleen cells fused according to the schedule in Figure 2. Primary response mice received a single injection of 100 μg So-KLH in saline, i.v. Secondary response mice received a total of three immunizations of So-KLH separated by two-week intervals, the first in Complete Freund's Adjuvant (CFA, 100 μg i.p.), the second in Incomplete Freund's Adjuvant (IFA, 100 μg i.p.) and the last in saline (50 μg i.v.). Eight hybridomas were obtained from mice receiving two additional injections of So-KLH (100 μg i.p. and 50 μg i.v.) after a two month rest.

Spleen cells were removed four days after the last immunization in each group of 2-4 mice, pooled and fused with the non-secreting myeloma line FO as described previously (6). Hybridomas with supernatants positive for Soman-specific antibody by ELISA were cloned twice from single cells by the hanging drop method (14).

Soman analogs and hapten inhibitors:

Haptens used to define four major fine specificity groups included the following Soman derivatives: p-aminophenyl-Soman (NH_2-ϕ-So),

p-nitrophenyl-Soman (NO_2-ϕ-So) and dipinacolylmethylphosphonate (DPMP) were provided by Dr. D. Lenz (US-AMRICD, Aberdeen Proving Ground, MD). Hapten-protein conjugates used to inhibit IgM monoclonals included TNP_{16}-BSA, PC_{11}-BSA, So_{26}-BSA and So_{1356}-KLH.

Fine Specificity Analysis:

The fine specificity of Soman-specific antibodies was determined in an ELISA inhibition assay as described (6) on Soman-BSA-coated plates in the presence or absence of varying concentrations of hapten. The I_{50} value for each inhibitor was calculated and is expressed as the mM hapten concentration required for 50% inhibition of anti-Soman antibody binding to So-BSA.

DNA and RNA isolation:

DNA and RNA were isolated from anti-Soman hybridoma cells, and PCG1-1 and FO cells grown in tissue culture. TEPC15, aPC104-8, aPC52-1 and M104E cells were harvested as subcutaneous tumors grown in BALB/c mice. High molecular weight DNA and total RNA were prepared by a modified guanidinium thiocyanate/cesium chloride method (15).

Southern blot analysis:

Isolated DNA was digested with EcoRI, BamHI, HindIII or XbaI at 37°C, electrophoresed on 0.6% or 0.7% agarose/tris-acetate gels for 17-48 hours and transferred to Nytran membrane (Schleicher and Schuell, Inc., Keene, NH) according to Southern (16). Prehybridizations and hybridizations were performed at 42°C in 6X SSC (1X = 0.15 M sodium chloride, 0.015 M sodium citrate), 0.5% SDS, 5X Denhardt's solution (1X = 0.02% bovine serum albumin,

0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 50% formamide, 10% dextran sulfate, 0.01 M EDTA, and 200 µg/ml denatured salmon sperm DNA. Probes were labeled with ^{32}P-dATP to a specific activity of $8x10^8$ dpm/ug using random oligonucleotide primer extension (17) and hybridized at $3\text{-}4x10^6$ dpm/ml. Filters were washed in low stringency (3xSSC) and high stringency (0.2xSSC) buffers at 60^0 or 65^0C by the method of Brodeur and Riblet (18).

Northern blot anlaysis:

Ten µg of total RNA were electrophoresed on a 1% agarose/formaldehyde gel for 6 hrs and transferred to Nytran membrane according to Southern (16). RNA concentrations were determined beforehand by absorbance at 260 nm. Prehybridizations, hybridizations, probe labeling and washing of RNA blots were performed in the same manner as for Southern blots. Each Northern blot contained several control RNA's representing different VH families as well as FO RNA in order to ensure probe specificity.

Probes:

The following JH region probes were used in Southern blot analysis. A 3.2 kilobase pair (kbp) probe (pMJH) containing all four BALB/c JH genes cloned into PBR322 (19) was provided by Drs. R. Perlmutter and L. Hood. A 1.95 kbp HpaII-HpaII fragment of pMJH containing the JH coding sequences was used to probe EcoRI- and XbaI-digested DNA. The following probes were used in Northern analysis to detect members of specific VH gene families. A .98 kbp TaqI-TaqI fragment containing the coding region from the VH of J558 was derived from a probe (18) provided by Dr. R. Riblet and cloned into pUC18 for use in detecting J558 VH gene family members. S107 VH gene family usage was determined using a probe provided by Dr. R. Perlmutter containing 445 bp of

the rearranged V-D-J genes from S107 (20). Q52 VH gene family usage was detected using a probe containing the VH from PCG1-1 which uses the M141 VH gene (21). The 36-60 (22), 7183 (23) and X24 (24) family probes were kindly provided by Drs. M. Gefter, F. Alt, and S. Rudikoff, respectively. The Jκ probe was a 600 bp AccI-XbaI fragment containing Jκ5 derived from pVκ1-3 (25) provided by Dr. J. Kenny. A 5.0 kbp Vκ1 probe was also derived from pVκ1-3; it consisted of approximately 190 bp of the Vκ1A coding region plus approximately 4.3 kbp of it's 5' flanking sequence.

RESULTS

Hybridoma production:

All hybridomas generated after one injection of So-KLH produced IgM antibodies. These mAbs were shown to bind to So-BSA and So-KLH-coated ELISA plates but not to BSA, KLH, PC-BSA or TNP-KLH coated plates (not shown). In addition, the majority of these mAbs (6 of 8) were inhibitable in an ELISA inhibition assay only by protein-conjugated forms of Soman but not by free hapten analogs of Soman (Table I). Protein carriers conjugated with control haptens such as PC or TNP failed to inhibit mAb binding to So-BSA demonstrating that these hybridomas were indeed producing Soman-protein-specific antibody.

The majority of hybridomas generated in the secondary and tertiary response fusions produced mAb of the IgG isotype. The monoclonals which were further characterized include 18 IgG1, 7 IgG2a, 3 IgG2b, 5 IgG3 and 5 IgM. The IgM mAbs from these fusions were shown to bind specifically to Soman but 4/5 were not inhibited by free hapten, i.e. they were comparable to the IgM monoclonals obtained during the primary response (Table I). In contrast, all IgG mAbs were inhibitable by at least one hapten analog of Soman (Table I).

Four fine specificity groups are defined by inhibition ELISA:

The fine specificity of Soman-specific mAbs was first assessed by the ability of three Soman analogs, DPMP, NO_2-ϕ-So and NH_2-ϕ-So, to inhibit binding to So-BSA. DPMP more closely resembles the Soman molecule itself whereas NO_2-ϕ-So and NH_2-ϕ-So more closely resemble the immunizing form of Soman when it is coupled to KLH via a diazophenyl linker. We detected four

main fine specificity groups based on this analysis (Table I). Hybridomas belonging to Group A were shown to be inhibited by all three of these Soman analogs. Group B hybridomas were inhibited only by the phenyl-containing NO_2-ϕ-So and NH_2-ϕ-So derivatives. The hybridomas in Group C demonstrated measurable inhibition by NH_2-ϕ-So alone. As indicated above, the majority of IgM hybridomas appear to be specific for Soman-protein only and could not be characterized by hapten inhibition; consequently they are placed in a separate specificity group designated Group D. Serum responses to Soman-KLH in CFA through day 14 consist primarily of IgM antibodies which are not inhibitable by free Soman hapten, thus they resemble Group D antibodies (not shown).

Predominant use of the J558 VH gene family among Soman-specific hybridomas:

In order to determine VH gene usage, total RNA from each of the 46 hybridomas was isolated and screened by Northern blot analysis for hybridization to probes specific for six of the known VH gene families. The results of these experiments (see Table I) demonstrated predominant use of VH J558 gene members among the IgG hybridomas (25/33). VH J558 is also represented in 7/13 IgM hybridomas. VH 7183, VH 36-60 and VH Q52 also contribute to the Soman-specific response although to a much lesser degree. Two hybridomas remain unidentified with respect to VH family usage as they were negative for all VH probes tested including those for VH S107 and VH X24. It is apparent from these data that VH J558 is used significantly by hybridomas in all fine specificity groups.

Soman-specific antibodies are encoded by several distinct VH genes:

The high proportion of VH J558-positive clones raised the possibility that one or a few J558 genes are used preferentially to create the anti-Soman combining site. The other possibility is that J558 predominance correlates with the relative size of this large VH family. The estimated number of J558 gene members (500-1000, (26)) as well as the extreme homology which may exist between family members make it difficult to attribute a common-sized JH rearrangement to shared use of a single VH gene. In fact, a subset of 12 closely related J558 VH genes which show homology extending several kilobases into the flanking regions has been described (27). However, the minimum number of distinct VH J558 genes utilized may be determined by comparing the relative sizes of JH rearrangements while keeping in mind that small size differences may be attributable to use of the same VH gene with different JH segments. Consequently, DNA was isolated from each hybridoma and digested with EcoRI or XbaI and analyzed for JH rearrangement by probing with pMJH or a 1.95 kbp fragment containing the JH coding regions.

Analysis of the JH rearrangements among the $J558^+$ IgM hybridomas in this manner indicated that a minimum of 5/7 utilize different VH genes (Table II). Among the 25 $J558^+$ IgG hybridomas there are at least ten different J558 VH genes in use (i.e. restriction fragment differences that could not be ascribed to differential JH usage, Table II). Similar results were obtained for 7183- and 36-60-positive hybridomas where more than one member from each of these families appears to be used (Table II). Thus, it seems that Soman-specific antibodies can be encoded not only by a number of distinct VH gene families but also by a number of different VH genes within a given family.

These results are not indicative of preferential VH gene usage but rather they suggest unrestricted usage.

Vκ1 usage by Soman-specific hybridomas:

The Vκ1 family has been demonstrated to play a significant role in the secondary response to PC-KLH (7-9). Here, we show that Vκ1 also plays an important role in the antibody response to Soman-KLH. Hybridization of Northern blots with a Vκ1 probe demonstrated that 18 hybridomas from different fine specificity groups produced $V\kappa1^+$ transcripts (Table I). Restriction digests of the 18 anti-Soman-KLH hybridomas were compared to those of 3 $V\kappa1^+$, PC-KLH-specific hybridomas (aPC104-8, PCG1-1 and aPC52-1) whose light chains have been sequenced (Stenzel-Poore et al, in preparation): Eleven of the anti-Soman-KLH hybridomas shared with PCG1-1 (Vκ1C-Jκ2) an approximately 3.4 kbp HindIII rearrangement which hybridized to both the Vκ1 and Jκ probes (Figure 3; see also Table IIIA1). However, further analysis with XbaI digestion detected a unique 8.7 kbp $V\kappa1^+$ $J\kappa^+$ rearrangement in the 11 anti-Soman-KLH hybridomas absent in PCG1-1 (Figure 4; Table IIIA2) indicating that the anti-Soman-KLH hybridomas were unlikely to be using Vκ1C. Another hybridoma, SoM-10, shared an 11.5-11.6 kbp $V\kappa1^+$ $J\kappa^+$ BamHI fragment with aPC104-8 (Vκ1C-Jκ1, Table IIIB1) but XbaI digestion yielded a 9.1 kbp $V\kappa1^+$ $J\kappa^+$ fragment approximately 1 kbp smaller than that of aPC104-8 (10.1 kbp, Table IIIB2) indicating that SoM-10 was also unlikely to be using Vκ1C. Five of the 6 remaining anti-Soman-KLH hybridomas shared a $V\kappa1^+$ $J\kappa^+$ BamHI fragment (10.3 kbp, Table IIIB1) that was 550 bp larger than that of aPC52-1 (Vκ1A-Jκ5, 9.75 kbp); the same 5 hybridomas also shared an 8.05 kbp $V\kappa1^+$ $J\kappa^+$ XbaI fragment (Table IIIB2) that was approximately 400 bp larger than the 7.65

kbp fragment of aPC52-1. HindIII digests from the same 5 hybridomas did not result in detectable Vκ1$^+$ Jκ$^+$ rearrangments, possibly due to comigration with a germline restriction fragment (not shown).

Vκ1A and Vκ1C genes share a high degree of homology extending into the 5' flanking region (11,12). As a result, HindIII or BamHI digestion of a Vκ1-Jκ rearrangement does not distinguish between the use of Vκ1A or Vκ1C. However, XbaI digestion yields a Vκ1C-Jκ rearrangement approximately 1 kb larger than a Vκ1A-Jκ rearrangement using the same Jκ segment. Collectively, our restriction analysis comparing HindIII, BamHI or XbaI digestion is consistent with rearrangement of a Vκ1 gene other than Vκ1C in 17/18 Vκ1$^+$ anti-Soman-KLH hybridomas. Preliminary sequencing analysis of mRNA from SoG1-1, SoG1-9, SoG1-10, SoG1-12 and SoG2a-1 indicates that these hybridomas utilize a Vκ1 gene most homologous to Vκ1A rearranged to Jκ2 (A.B., unpublished). The known distances between various Jκ segments (28) are consistent with the differences between fragment sizes presented in Table III. Thus it is possible that all 17 hybridomas use the same Vκ1 gene rearranged to different Jκ segments. In contrast sequence analysis suggests that SoG2b-1 uses a Vκ1 gene most homologous to Vκ1C also rearranged to Jκ2 and this is consistent to what has been observed by restriction analysis (A.B., unpublished).

DISCUSSION

The pattern of inhibition seen with the Group A mAbs suggests that they require a minimal recognition structure comprised of the terminal six-carbon pinacolyl group which is shared among the haptens DPMP, NO_2-ϕ-So and NH_2-ϕ-So. Brimfield et al, (29) also showed that this region was important in the specific binding of two Soman-specific mAbs by using analogs in which the pinacolyl structure was systematically altered. The importance of the phenyl moiety is less clear in Group A binding although it seems to be recognized since the phenyl-containing haptens NO_2-ϕ-So and NH_2-ϕ-So were more inhibitory compared to DPMP for many Group A antibodies (Table I).

In contrast the phenyl moiety appears to play a primary role in recognition by the Group B mAbs since they are inhibited only by phenyl-containing haptens. Group C mAbs have fine specificities very similar to those in Group B, i.e. the phenyl region appears to be a dominant feature of the epitope but inhibition by NO_2-ϕ-So is not demonstrable in Group C (Table I). We have found that the majority of anti-Soman-KLH hybridomas (Group D) are inhibitable by protein-coupled forms of Soman but not by free hapten derivatives of Soman. One possible explanation is that the Group D hybridomas produce antibodies of low affinity such that free Soman hapten does not compete effectively with the binding of IgM antibodies to a multivalent Soman-conjugate-coated surface. Alternatively, the IgM monoclonal antibodies could require a much larger structure than the Soman molecule alone or its haptenic analogs in order to bind efficiently.

We initially questioned the existence of cross-reactivity among anti-Soman antibodies for PC analogs and vice versa since uncharged trimethyl-containing compounds including Soman are competitive inhibitors of

acetylcholinesterase (30) which ordinarily binds the charged trimethylammonium group of acetylcholine. Furthermore, Lenz et al, (31) showed that a Soman-specific mAb could compete with acetylcholinesterase for binding to Soman suggesting that the active sites of both antibody and enzyme may recognize a common feature(s). Some Group II anti-PC-KLH antibodies have been shown to bind an analog of nitrophenylphosphocholine in which the positively-charged nitrogen was replaced by a carbon atom (32). However such antibodies do not appear to bind Soman (6; U. Bruderer, unpublished) which is consistent with the view that the negative charge of the phosphate moiety is also a critical feature in the binding of anti-PC-KLH mAbs but not of anti-Soman antibodies; most Soman-specific antibodies are inhibited by analogs bearing either an uncharged or negatively charged phosphoryl group but none are able to recognize analogs bearing a positively charged choline group (6; unpublished). Analysis of fine structure recognition among Soman-specific mAbs suggests that the phenyl moiety is an immunodominant feature of the immunogen, So-KLH. In fact, this was found to be the case with polyclonal antisera raised in rabbits (31) and mice (A.B., unpublished) also immunized with So-KLH. Comparable conclusions have been reached in the secondary anti-PC-KLH response where NPPC has been found to be a much better inhibitor of IgG antibodies than PC (4,33).

No apparent correlation was found between the VH or VL families being used and fine specificity phenotype (Group A, B, C or D). VH gene analysis revealed a predominance of J558 family members while a smaller number of clones represented the 7183 and 36-60 families. Dildrop et al. (34) demonstrated that VH gene expression in hybridomas generated after LPS stimulation also showed J558 predominance and that distribution of VH family occurrence appeared to correlate with family size suggesting random VH gene

usage. It is possible that the high frequency of J558$^+$ anti-Soman hybridomas is also due to the large size of this family rather than to preferential gene usage. In fact, we have found that at least ten different members of the J558 family participate in this response. However, at present we cannot exclude the possibility that these 10 or more members represent a small subset and therefore that the anti-Soman response is restricted within VH J558.

We found that 18/46 anti-Soman hybridomas utilized the Vκ1 family. The remaining hybridomas are unidentified with respect to VL usage. Restriction analysis suggested that 17/18 Vκ1$^+$ hybridomas are not using the Vκ1C gene. Preliminary sequencing data show that at least 5 use Vκ1 genes most homologous to Vκ1A (unpublished). As mentioned earlier, the Vκ1 family plays a significant role in the secondary response to PC-KLH (7,8). In contrast to the anti-Soman hybridomas, it was the Vκ1C gene that was used by 6/7 anti-PC hybridomas (9). While it is clear that other VH and VL combinations are capable of generating Soman-phenyl combining sites, we and others (9,35) have proposed that Vκ1 members may contribute to the formation of combining sites which bind well to phenyl-containing structures. We suggest that different members of this family may distinguish between spatially similar but chemically distinct structures (i.e. Soman and PC). Interestingly, Corbet et al, (12) found that the Vκ1A gene is used by most anti-GAT hybridomas while the Vκ1C gene is used by most hybridomas which recognize an "internal image" of GAT borne by anti-idiotypic antibodies of the GAT idiotypic cascade. It has been suggested (36) that tyrosine and glutamic acid residues within the D region of these anti-id antibodies mimic a dominant GAT epitope. This would be consistent with the view that Vκ1 is important in the recognition of phenyl-containing structures. In addition, these findings also support the view that Vκ1A and Vκ1C-encoded light chains can distinguish

between two similar but non-identical structures. Other studies have also described the use of Vκ1 genes in antibodies binding aromatic or ring-bearing structures such as phenyloxazolone (10), DNA (13) and the branched complex T,G-A--L (11).

It has been suggested that the role of the light chain may be more significant than that of the heavy chain in binding to some epitopes (37,38). The anti-Soman-KLH monoclonals described here utilize a number of different VH genes (as does the anti-PC-KLH response (9)) suggesting non-restricted choice of VH. In contrast there may be more restricted use of VL genes. At least 25 Vκ families have been described (39) of which Vκ1 is one of the smaller (40) and does not appear to be proximal to Jκ (41). In addition, both Vκ1A and Vκ1C have been reported to participate in several other responses (35) although among the anti-Soman hybridomas there may be a preferential choice of Vκ1A over Vκ1C.

Finally, we note that while the initial response to PC-KLH is highly restricted to antibodies bearing the T15 idiotype (42,43), the anti-Soman-KLH response begins and apparently continues as a heterogeneous response. It has been proposed that restriction in the anti-PC response has evolved as a result of the ubiquitious nature of phosphocholine and the fact that $T15^+$ anti-PC antibodies provide effective protection against PC-bearing bacteria (44,45). The heterogeneity seen throughout the response to Soman may reflect the fact that Soman is not an environmental antigen. In the absence of any evolutionary pressure to establish one particularly efficient immune response in the germline (as may have occurred with $T15^+$ anti-PC), it may not be surprising that a diverse collection of B cell clonotypes responds to immunization with Soman. It will be interesting to determine whether boosting with Soman after a long period of rest will give rise to a more limited

number of clonotypes that predominate in the late memory response or whether the heterogeneous pattern that was established in the primary and secondary responses is continued in the pool of memory B cells.

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Table I: FINE SPECIFICITY AND V GENE USE OF ANTI-SOMAN HYBRIDOMAS

GROUP A HYBRIDOMAS

			I50 (mM)[b]			gene usage[c]	
CLONE	fusion[a]	isotype	NH_2So	NO_2So	DPMP	VH	Vk1
SoM-4	2°	μ,λ	0.0002	0.14	1	J558	-
SoG2a-1	3°	γ2a,κ	0.033	0.033	1	J558	+
SoG2a-3	2°	γ2a,κ	0.001	0.015	0.9	J558	+
SoG2a-4	2°	γ2a,κ	0.0004	0.02	0.24	J558	+
SoG2a-5	2°	γ2a,κ	0.00014	0.007	0.1	J558	+
SoG2a-6	2°	γ2a,κ	0.0009	0.026	0.6	J558	+
SoG2a-7	2°	γ2a,κ	0.04	0.18	0.7	J558	-
SoG1-2	2°	γ1,κ	0.01	0.12	0.08	J558	-
SoG1-3	2°	γ1,κ	0.009	0.26	0.004	U[d]	+
SoG1-4	2°	γ1,κ	0.0005	0.93	0.9	J558	+
SoG1-5	2°	γ1,κ	0.0003	0.002	1	U	+
SoG1-6	2°	γ1,λ	0.0003	0.15	0.7	J558	-
SoG1-7	2°	γ1,κ	0.004	0.19	1	J558	-
SoG3-1	3°	γ3,κ	0.06	0.13	0.32	36-60	-
SoG3-2	3°	γ3,κ	0.06	0.23	0.09	J558	-
SoG3-3	3°	γ3,κ	0.06	0.27	0.09	J558	-
SoG3-4	2°	γ3,κ	0.03	0.14	0.43	J558	-

GROUP B HYBRIDOMAS

			I50 (mM)[b]			gene usage[c]	
CLONE	fusion[a]	isotype	NH_2So	NO_2So	DPMP	VH	Vk1
SoM-6	1°	μ,k	0.013	0.06	>1	36-60	+
SoM-9	1°	μ,λ	0.02	0.3	>1	J558	-
SoG2a-2	3°	γ2a,κ	0.018	0.0008	>1	36-60	-
SoG2b-1	3°	γ2b,κ	0.02	0.035	>1	7183	+
SoG1-1	3°	γ1,κ	0.002	0.0004	>1	J558	+
SoG1-8	2°	γ1,κ	0.0007	0.7	>1	J558	-
SoG1-9	2°	γ1,κ	0.024	0.13	>1	J558	+
SoG1-10	2°	γ1,κ	0.002	0.015	>1	J558	+
SoG1-12	2°	γ1,κ	0.004	0.2	>1	J558	+
SoG1-13	2°	γ1,λ	0.0003	0.26	>1	J558	-

GROUP C HYBRIDOMAS

			I50 (mM)[b]			gene usage[c]	
CLONE	fusion[a]	isotype	NH_2So	NO_2So	DPMP	VH	Vk1
SoG2b-2	2°	γ2b,κ	0.18	>1	>1	J558	-
SoG2b-3	2°	γ2b,κ	0.03	>1	>1	7183	-
SoG1-11	2°	γ1,κ	1	>1	>1	Q52	-
SoG1-14	2°	γ1,κ	0.02	>1	>1	36-60	+
SoG1-15	2°	γ1,κ	0.34	>1	>1	J558	-
SoG1-16	2°	γ1,κ	0.22	>1	>1	J558	-
SoG1-17	2°	γ1,κ	0.23	>1	>1	J558	-
SoG1-18	2°	γ1,κ	0.1	>1	>1	J558	-
SoG3-5	2°	γ3,κ	0.37	>1	>1	J558	-

GROUP D HYBRIDOMAS

				I50 (mM)[b]		gene usage[c]	
CLONE	fusion[a]	isotype	hapten[e]	SoKLH	KLH	VH	Vk1
SoM-1	3°	μ,κ	>1	0.0017	--[f]	7183	-
SoM-2	2°	μ,κ	>1	0.0012	- -	7183	-
SoM-3	2°	μ,κ	>1	0.00001	- -	36-60	-
SoM-5	2°	μ,κ	>1	0.00001	- -	J558	+
SoM-7	1°	μ,κ	>1	0.00006	- -	J558	+
SoM-8	1°	μ,λ	>1	0.00016	- -	7183	-
SoM-10	1°	μ,κ	>1	0.002	- -	J558	+
SoM-11	1°	μ,κ	>1	0.00003	- -	J558	-
SoM-12	1°	μ,κ	>1	0.00006	- -	J558	-
SoM-13	1°	μ,κ	>1	0.00001	- -	36-60	-

[a] See Figure 2 for immunization and fusion time.

[b] mM hapten inhibiting to 50% binding in ELISA assay.

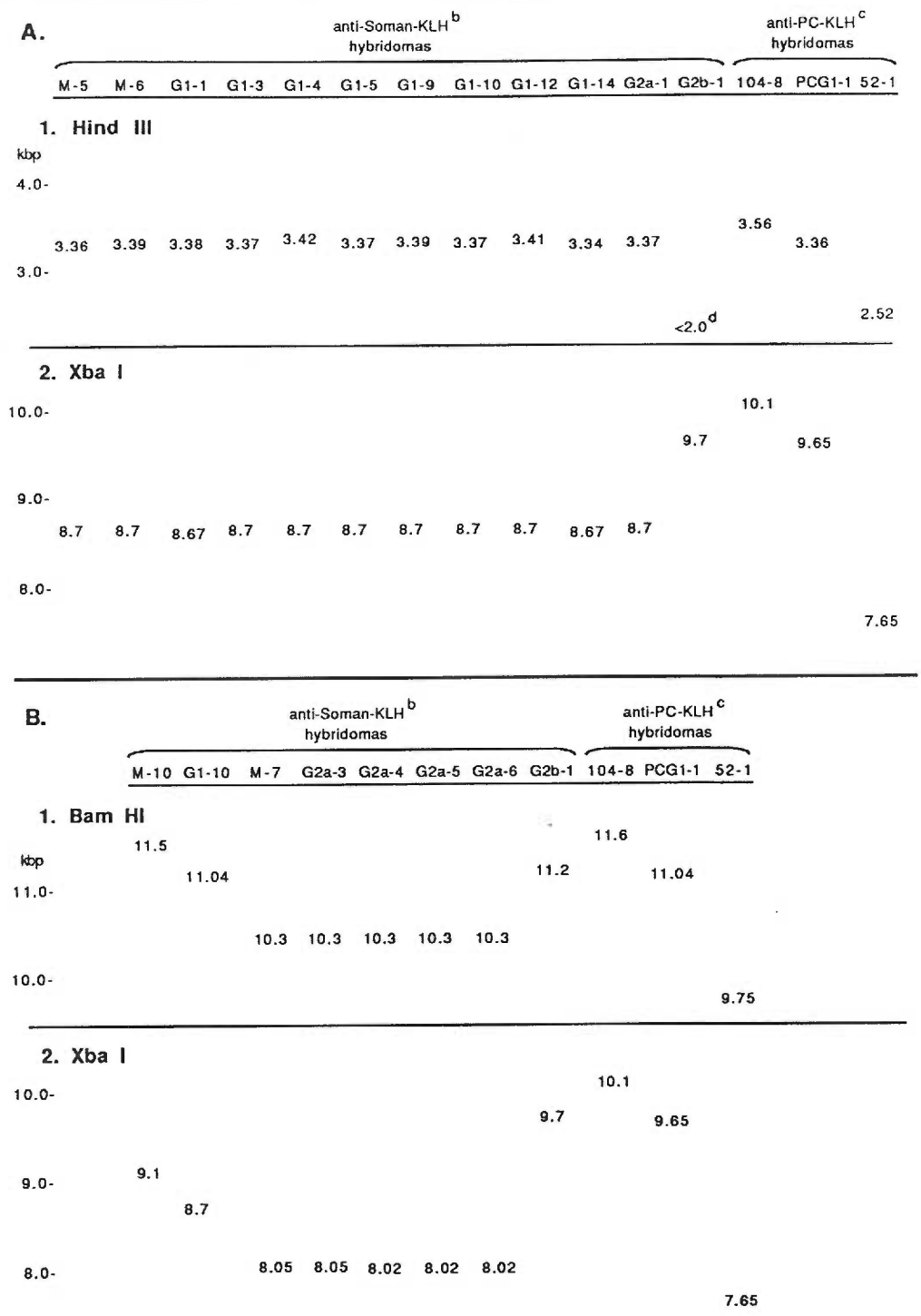
[c] V gene usage determined by Northern blot analysis.

[d] U = unidentified

[e] $NH_2\phi So$, $NO_2\phi So$ and DPMP

[f] -- indicates no inhibition at 1 mg/ml KLH

Table III: Restriction analysis of productive Vk1 rearrangements in anti-Soman-KLH hybridomas compared to three anti-PC-KLH hybridomas.[a]



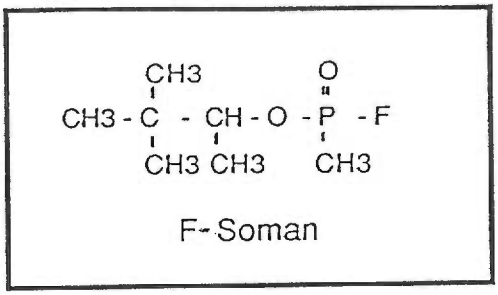
[a] DNA samples were digested with Hind III, Bam HI or Xba I and size separated in 0.6 or 0.7% agarose; Southern blots were hybridized with Vk1 and Jk probes to identify the productive rearrangements shown.

[b] Isotype, fine specificity and V gene usage are provided in Table I.

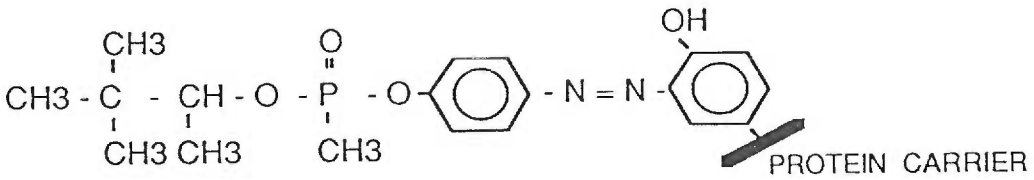
[c] The following assignments have been derived from nucleotide sequencing data (Stenzel-Poore et al., in preparation): aPC104-8 (Vk1C-Jk1); PCG1-1 (Vk1C-Jk2); aPC52-1 (Vk1A-Jk5).

[d] Hybridization with Vk1 detected a 1.6 kbp rearrangement while Jk hybridization detected a 1.9 kbp rearrangement.

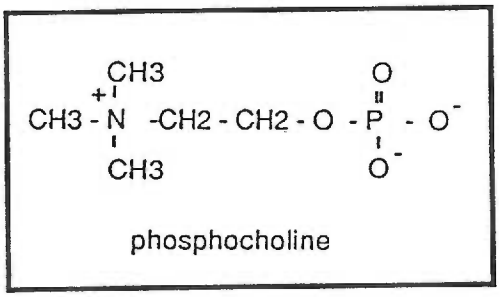
Figure 1: Structure of the haptens Soman and PC (see insets) when coupled to a protein carrier.



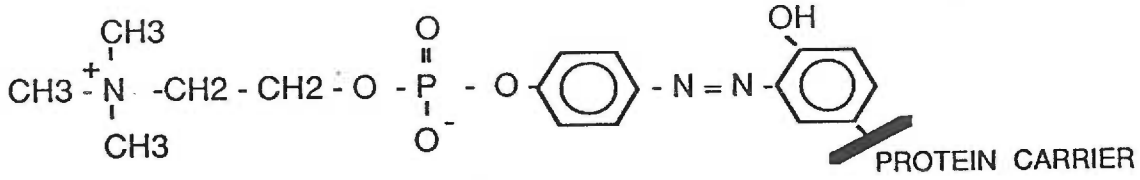
F-Soman



Soman-Protein



phosphocholine



PC - Protein

Figure 2: Schedule of immunization(s) prior to fusions.

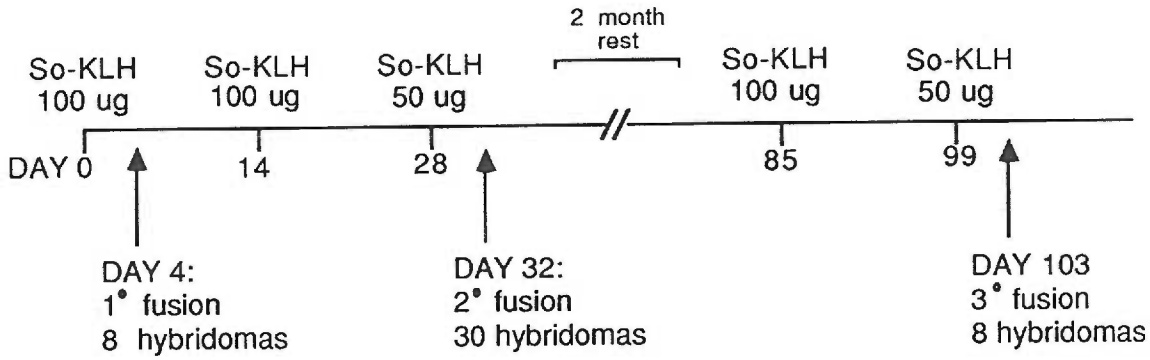

2 month
rest
So-KLH
100 ug
So-KLH
100 ug
So-KLH
50 ug
So-KLH
100 ug
So-KLH
50 ug
DAY 0
14
28
85
99
DAY 4:
1° fusion
8 hybridomas
DAY 32:
2° fusion
30 hybridomas
DAY 103
3° fusion
8 hybridomas

Figure 3: A 3.4 kbp HindIII rearrangement is detected using a Jκ probe (left panel) and a Vκ1 probe (right panel) in a total of 11 $V\kappa1^+$ anti-Soman-KLH hybridomas (only 4 are shown) and in PCG1-1, a $V\kappa1^+$ anti-PC-KLH hybridoma. FO, the mycloma fusion partner, contributes 1 or 2 Jκ bands to each anti-Soman hybridoma.

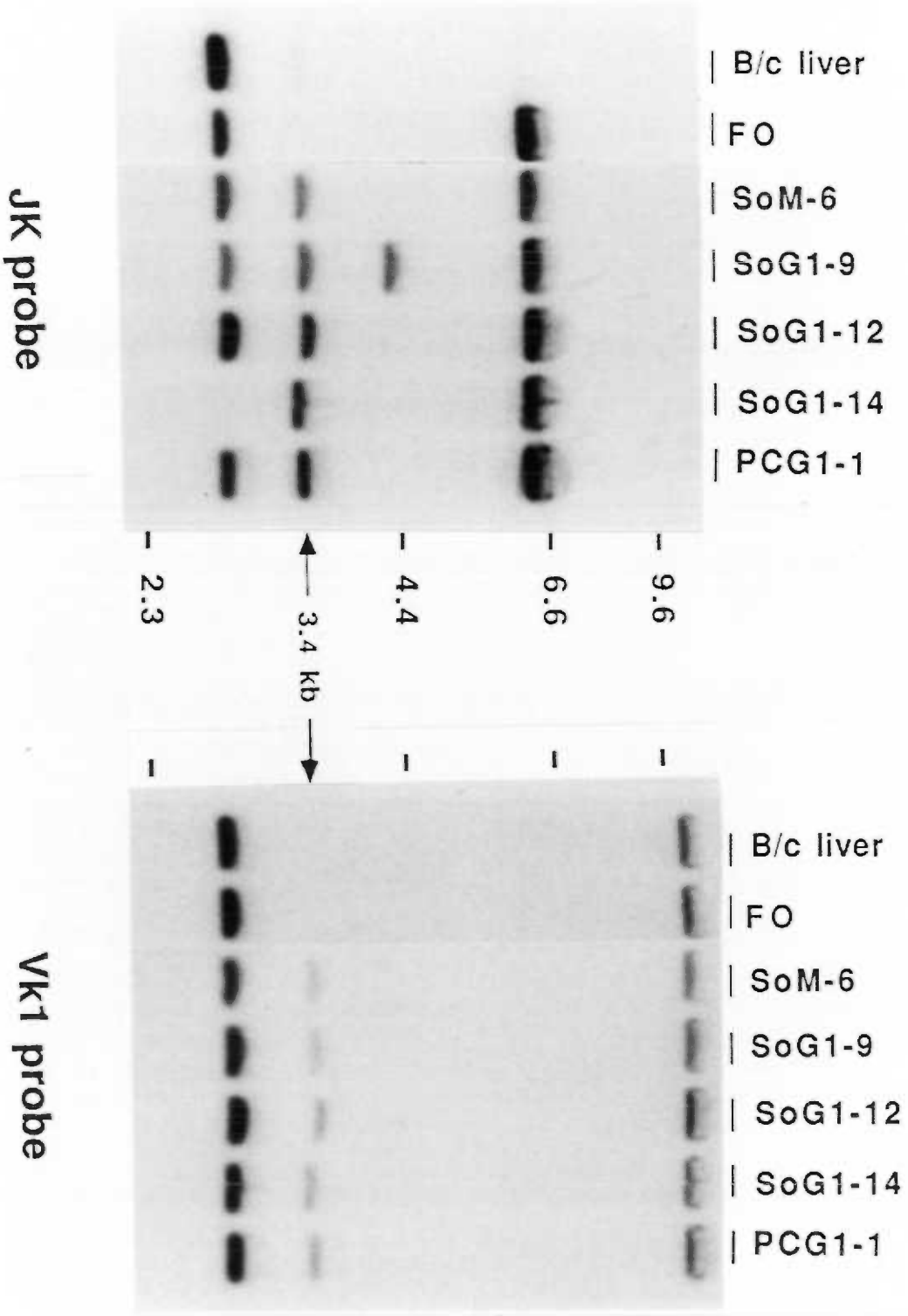

JK probe
B/c liver
FO
SoM-6
SoG1-9
SoG1-12
SoG1-14
PCG1-1
2.3
3.4 kb
4.4
6.6
9.6
Vk1 probe
B/c liver
FO
SoM-6
SoG1-9
SoG1-12
SoG1-14
PCG1-1

Figure 4: An 8.7 kbp XbaI rearrangement is detected with a Jκ probe (left panel) and a Vκ1 probe (right panel) in a total of 11 Vκ1+ anti-Soman-KLH hybridomas (only 6 are shown). PCG1-1, a $V\kappa1^+$ anti-PC-KLH hybridoma, has a 9.65 kbp $V\kappa1^+$ $J\kappa^+$ XbaI rearrangement which is obscured in the right panel by a germline Vκ1 band but can be detected with a Vκ1 probe after an extended gel separation time (not shown).

JK probe
FO
SoG1-8
SoG2a-1
SoM-5
SoG1-9
SoG1-12
SoG1-14
SoG1-1
PCG1-1
9.6
8.7 kb
6.6
4.4
Vk1 probe
FO
SoG1-8
SoG2a-1
SoM-5
SoG1-9
SoG1-12
SoG1-14
SoG1-1
PCG1-1

Paper 3

Proposed Combining Site Models for the Binding of Organophosphorus Haptens

Abigail C. Buenafe, Mary P. Stenzel-Poore and Marvin B. Rittenberg

INTRODUCTION

Specific B cell activation and memory development are dependent upon the interaction of antigen with immunoglobulin receptors on the B cell surface. The nature of this interaction is important since it is a major means by which selective mechanisms shape the responsive memory B cell population (1-3). We are investigating the murine immune response to the organophosphorus neurotoxin Soman [O-1,2,2 trimethylpropylmethylphosphonofluoridate] as a model system. A panel of 46 hybridomas was generated against Soman coupled to keyhole limpet hemocyanin (So-KLH) and analyzed for fine specificity and V gene use (4,5). A subset of these hybridomas demonstrated restricted use of the Vk1 family in combination with different VH genes. We are also studying the antibody response to another organophosphorus hapten, phosphocholine-KLH (PC-KLH). The 2 haptens Soman and PC are similar in shape and structure but possess significant charge differences which are likely to account for the lack of cross-reactive antibody (4,5). By comparing these responses we hope to gain an understanding of the molecular basis for specific recognition of these organophosphorus haptens. V_H usage is diverse in the secondary response to PC-KLH (6-8) yet a significant portion of these antibodies also use Vk1(7-10). Interestingly, a different Vk1 member predominates in each of these responses (5,7,8).

Here we report the V_L and V_H sequences derived from mRNA sequencing of 9 Vk1+ anti-So-KLH hybridomas. We have analyzed these sequences and compared them to those obtained previously from 8 Vk1+ anti-PC-KLH hybridomas. Potential structural elements which could contribute to hapten binding in these 2 sets of mAbs recognizing related but charge-dissimilar

haptens are discussed in light of binding site models (11-14) based on the atomic coordinates of several crystallized V domain structures.

MATERIALS and METHODS

Hybridomas:

Generation and cloning of Soman-KLH-specific hybridomas have been described elsewhere (4,5).

mRNA sequencing:

Total RNA was isolated from hybridoma cells grown in tissue culture as described (5). PolyA+ mRNA was isolated by passage over oligo(dt)cellulose (New England Biolabs) and quantified by absorbance at 260nm. Sequencing was performed by reverse transcription using ^{32}P-labeled synthetic primers (15). PolyA+ RNA (5.0 ug) was reverse transcribed in the presence of 2500 μM dGTP, dATP, dTTP and dCTP plus one of the following: 100 μM ddGTP, 150 μM ddATP, 250 μM ddTTP, or 150 μM ddCTP.

Oligonucleotide primers:

Synthetic primers used in sequencing reactions included primers specific for the μ, γ1, and κ constant regions: μ primer = 5' GCTCTCGCAGGAGAC 3' (16); γ1 primer = 5' GGGGCCAGTGGATAGAC 3' (17); κ primer = 5' TGGATGGTGGGGAGATG 3' (17). V region primers specific for the internal frameworks of VHJ558, VH7183 and Vk1 family members were also used: J558 = 5' GCAGAGTC5TCAGATGTC 3' (18); 7183 = 5' TCTTGCACAG7AATA 3'(19); Vk1 = 5' CTGTCCCTGATCCACTGCCACT 3' (20).

Sequence analysis:

The sequences discussed here are numbered according to Kabat et al. (21). The hypervariable regions designated by Chothia and coworkers (12) differ

from the more extensive complementarity determining regions described by Kabat et al. (21). We have elected to use the nomenclature of Chothia et al. in the discussion of proposed combining site models based on known canonical structures (11,12).

Sequence manipulation, structural analysis and homology searches were performed using the BIONET Resource computer facility (Palo Alto, CA). Prediction of secondary structure from derived amino acid sequences was accomplished using the method of Chou and Fasman (22) (BIONET Resource program).

Surface variability plots were generated using the method described by Kiebber-Emmons et al. (23). Surface variability is calculated as the product of variability in hydropathicity at a given amino acid position and the average hydropathicity at that position. Hydropathicity values (24) (BIONET Resource program) for each protein were calculated across 7 residues with the average assigned to the central position. Variability in hydropathicity was calculated according to the method of Kiebber-Emmons et al. (23) and is based on the Wu and Kabat formula for calculating amino acid variability (25), i.e. the number of different hydropathicity values at a given residue was divided by the frequency of the most common value at that residue.

RESULTS and DISCUSSION

Anti-Soman-KLH Vk Sequences

Figure 1 shows the light chain nucleotide sequences and derived amino acid sequences of 9 Vk1+ anti-So-KLH hybridomas. The germline sequences of Vk1A and Vk1C differ at 10 nucleotides and are 94% homologous at the amino acid level (20). Consistent with restriction fragment analysis (5), 8 hybridomas were most homologous to Vk1A and shared 9/10 (SoG1-12, SoG2a-1) or 10/10 (SoM-5, SoM-7, SoM-10, SoG1-9, SoG1-10, SoG1-1) of the definitive nucleotides with Vk1A (Figure 1). In contrast, SoG2b-1 was most homologous to Vk1C with which it shared 9/10 definitive nucleotides (Figure 1). Also consistent with restriction fragment data was the fact that 7 hybridomas productively rearranged a Vk1 gene to Jk2, 1 to Jk1 and 1 to Jk4.

In light of a preference for Vk1A usage in the anti-So-KLH response, it should be noted that 2 potentially significant amino acid differences exist between germline Vk1A and Vk1C; these are His34 and Ser89 in Vk1A versus Glu34 and Phe89 in Vk1C (20). SoG2b-1 has retained Glu34 in CDR1 indicating that the presence of a large, acidic residue at this position is not sufficient to prevent So-KLH binding. The change from Phe89 to Leu89 in SoG2b-1, although not dramatic, may be significant as discussed below. Of the other 5 IgG hybridomas, only 2 possess amino acid changes in any light chain CDR: SoG1-12 has a conservative change from Tyr32 to Phe32 and SoG2a-1 has a conservative change from Ser26 to Gly26 as well as a change from His27d to the less basic Asn27d. All 3 changes occur in CDR1. Mutations among the 3 IgM hybridomas were found only with SoM-10 which has 3 replacement mutations and one silent mutation.

Mutational Distribution in Anti-So-KLH Vk

Table I summarizes the distribution of mutations leading to replacement versus silent changes in the 9 Vk1 light chains sequenced. In general, mutation among these IgG light chains appears to be distributed randomly among the 4 framework regions, CDR1, and CDR3. Mutation is absent in CDR2 of all the IgG hybridoma light chains. The ratio of replacement to silent (R:S) changes may indicate the results of selection for or against mutational change (26,27). For example, the required conservation of the overall structure of antibody molecules results in selection against changes in antibody framework regions. Consequently, R:S values calculated for framework regions are relatively small (generally 1.5 or less)(26). As shown in Table I, this is also true for the 6 IgG anti-So-KLH light chains sequenced where the majority of R:S values are less than 1. On the other hand, antigen selection for mutational change may be reflected by a greater number of replacement to silent mutations (>3) in the CDRs (26,27). As mentioned above, very few replacement changes were found in the light chain CDRs of anti-Soman-KLH hybridomas. In fact, the R:S values for 5/6 of the IgG hybridomas were less than or equal to 1, similar to R:S values found for the framework regions. The R:S ratio was greater than 1 only in SoG2b-1 (5 replacement to 3 silent changes) which is unique among anti-So-KLH hybridomas in its use of Vk1C. Moreover, 4/5 replacement mutations in SoG2b-1 were located in CDR3. This atypical distribution of mutations in Vk of SoG2b-1 may suggest that alteration of the Vk1C germline CDR3 sequence is necessary for the binding of So-KLH. On the other hand, the relatively few mutations associated with Vk1A light chains suggests that Vk1A germline sequences are already suitable for binding So-KLH.

Anti-So-KLH VH Sequences

Of the 9 hybridomas with V gene sequences reported here, 8 utilize VH genes belonging to the J558 family while one, SoG2b-1, uses a member of the 7183 family (28) (Figure 2). These findings are consistent with results from Northern blot analysis (5). Homology search and examination of the individual J558 VH sequences suggested that the majority are derived from different germline genes. One exception may be the J558-positive VH genes of SoM-10 and SoG1-10 which were found to share at least 93% and 97% homology, respectively, with the mRNA-derived VH sequence of an α1,6 dextran-specific hybridoma, 19.1.2 (29) (Figure 2). Southern blot data of SoM-10 and SoG1-10 VH (5) were consistent with their use of the same VH gene allowing for the use of J2 by SoM-10 and J3 by SoG1-10. SoM-5, SoG2a-1 and SoG1-9 share 89 - 92% homology with a different J558 gene but Southern blot data suggested that they do not use a common gene (5). The remaining 3 J558 VH sequences appeared to derive from 3 other J558 genes as determined by their overall sequence homologies (Figure 2) and Southern blot analysis (5). Therefore, these 9 anti-So-KLH VH sequences are likely to be derived from at least 8 different germline genes. Unfortunately, the large family size (29,30) and extreme homology that exists between different members of the J558 family (31) make it very difficult to assign a sequenced J558 VH to a particular germline gene. Consequently, analysis of the replacement and silent mutations which have occurred in VH is not possible here.

Sequence analysis:

Conservation of β-sheet framework:

Structural analysis of Ig V domains for which x-ray crystallographic data are available reveals a highly conserved framework consisting of 2 β-sheets packed face to face (11,32). Hypervariable regions which form the antibody combining site consist of one end of the β-sheets and the loops between adjacent or opposing β-strands (11,12). Figure 3a and 3b illustrate the regions of the V domain contributing to conserved framework or hypervariable regions. Analysis of predicted secondary structure from V region sequences of anti-So-KLH and anti-PC-KLH mAbs was performed in order to evaluate whether the β structure predicted in these mAbs would correlate with the β structure observed in known V domain structures. As a control, the same procedure was used to predict secondary structure from VH and VL sequences of myeloma protein M603, whose structure has been determined (33,34). Figure 3 summarizes the predicted secondary structure for 9 anti-So-KLH and 8 anti-PC-KLH VL and VH sequences and shows them to be dominated by β-strands at positions consistent with their contribution to the conserved framework. Secondary structure predicted by M603 VL and VH also correlated well with the observed framework although 2 extended regions in M603 VH (as well as FR4 in Vk of anti-So-KLH and anti-PC-KLH sequences) were more consistent with an α-helical conformation.

Hypervariable region conformations:

Most framework residues adjacent to hypervariable regions in 6 Ig V domains of known structure have been determined to vary by less than 1 A in position between various proteins (12). Analysis of the atomic coordinates of

these V domains led Chothia and Lesk to propose that the main chain conformation of hypervariable regions was determined by relatively few consensus residues located within the hypervariable regions and conserved framework (12). Comparison of these structurally important residues to those at homologous positions in anti-So-KLH and anti-PC-KLH sequences revealed a high degree of conservation as predicted by the Chothia-Lesk hypothesis. All L1 regions described here were more similar in length (12 residues) to that of M603 (13 residues) (12) than any other known structure with conserved residues shared at positions 25(Ser), 27b(Ile/Leu), and 33(Leu) (Figure 1, 4a). All L2 regions were identical in length (3 residues, Figure 1, 4a) as were all L3 regions (6 residues, Figure 1, 4b). The main chain of L2 packs against structurally conserved residues 48(Ile) and 64(Gly) (12), both of which are conserved in our sequences (see Figure 1). However, in the resolved structure of M603, the L2 region was prevented from making contact with bound hapten by the large L1 and H3 loops (33,35). L3 residue Pro95, also absolutely conserved in our sequences (Figure 4b), assumes a cis conformation in 2 known structures (M603 and REI)(12) causing an extension of residues 92 to 95. Amide side chains present at position 90 in the same structures (Asn in M603 and Gln in REI) form hydrogen bonds with main chain atoms at positions 93 and 95 (12). Thus it is noteworthy that Gln 90 is also conserved in all 17 Vk sequences discussed here (Figure 4b).

For the 7-residue H1 region (Figure 4c), conformationally important residues are conserved at positions 26(Gly), 29(Phe; Leu in one case), 34(Met, Ile; also Val, Leu) and 94(Arg or Lys, Figure 4d). H2 regions in 8/9 anti-So-KLH mAbs are 4 residues in length (Figure 4c) while the remaining H2 in SoG2b-1 is only 3 residues in length, comparable to the H2 length of anti-PC-KLH hybridoma PCG1-1. Anti-PC-KLH H2s vary from 3 to 6 residues in length. It

has been proposed that the exact conformation of a 4-residue H2 varies according to the positioning of Gly residues (12); thus with 1 or 2 Glys, the 4-residue H2's of anti-So-KLH and anti-PC-KLH antibodies could differ appreciably from one another while PCG2b-2, which has 3 Glys, may be unique. The larger 6-residue H2 regions may form an extended hairpin turn as found with M603 (12,33). The H3 regions in both sets of mAbs are highly variable in length (Figure 4d). 6/9 anti-So-KLH H3 regions were very small (3-4 residues) while the remaining 3 have H3 regions of 6, 8, or 13 residues. Such a small H3 region (3-4 residues) could significantly affect the size or shape of the antibody combining site. It is also possible that the diversity in H3 length signifies that the H3 loop does not play a major role in antigen contact among anti-So-KLH antibodies. Anti-PC-KLH H3 regions were less variable ranging from 6 to 10 residues in length. The H3 of M603 (9 residues) forms an extended hairpin loop determined mainly by the presence of a salt bridge between Arg94 and Asp101, as well as the participation of Tyr100b and Phe100c as interface residues between VH and VL (11,12). As discussed by Chien et al. (36), a salt bridge involving Arg94 of M603 is likely to be critical in binding the positively charged nitrogen of the PC hapten. In the anti-PC-KLH mAbs, at least 7/8 possessed the equivalent of Asp101 (taking into consideration that numbering in this region depends on length of the H3 loop) or a nearby Asp capable of interacting with Arg94 (Figure 4d) although since these antibodies do not bind appreciably to PC in the absence of the phenyl structure its relevance to Group II antibodies is not clear. The anti-So-KLH mAbs did not appear to have conserved such a residue which is consistent with their failure to bind to NPPC.

VL-VH interface residues:

Positioning of VL with VH may also affect the interaction of antigen with the combining site. The occurrence of preferential associations between certain sets of VL and VH chains has been suggested (37-39). One quarter of the VL-VH interface consists of residues contributed by hypervariable regions (11,13,14) and it is possible that these interacting residues control some aspects of H-L association and/or specificity for antigen.

Of the approximately 20 residues that form the VL-VH interface, 12 are absolutely or strongly conserved (11): VL 36, 38, 44, 87, and 98; VH 37, 39, 45, 47, 91, 93, and 103. These residues are likewise conserved in the anti-So-KLH and anti-PC-KLH mAbs. Variable interface residues are located within or adjacent to hypervariable regions (VL 34, 46, 89, 91, and 96; VH 35, 95, and 100) and are partially buried (11). The VH variable interface residues appear to be shared by both anti-So-KLH and anti-PC-KLH mAbs. VL interface residue Leu 46 is present in all sequences and VL position 96 is generally conserved as an aromatic residue.

In view of the preference for Vk1A usage among anti-So-KLH mAbs and for Vk1C usage among anti-PC-KLH mAbs (5,7,8), it is interesting to note that 3 of the 7 amino acid differences between Vk1A and Vk1C also participate as variable interface residues (the remaining 4 differences are highly conserved changes). Thus His34, Ser89, and Ser91 predominate in the panel of anti-So-KLH mAbs while Glu34, Phe89, and Gly91 predominate in the anti-PC-KLH mAbs (Figure 4a & b). An exception among anti-PC-KLH mAbs is 52-1 which uses the Vk1A light chain demonstrating that Vk1A usage is not absolutely excluded from the PC-KLH response. Also, two anti-PC-KLH mAbs, PCG1-14 and PCG1-2, use Vk24 light chains and have Asn34, Gln89, and Leu91. Of the 9 Vk1+ anti-So-KLH mAbs, the only exception which uses the Vk1C light chain is

SoG2b-1. Interestingly, SoG2b-1 has substituted Leu89 for Phe89. It is possible that one or more of these interface residues cause significant differences in VL-VH packing and subsequently influence the pairing of specific H and L chains or the overall conformation of the combining site. It is also possible for variable interface residues to influence specificity by contacting the antigen (33,40,41).

The antibody combining site:

The above analysis suggests that overall structure and packing of V domains in the 2 sets of mAbs are conserved and similar to that of known V domain structures. It was therefore considered reasonable to attempt identification of possible antigen-contacting residues within the combining site of these antibodies by modeling the hypervariable regions of anti-So-KLH and anti-PC-KLH mAbs after canonical structures present in these known V domains (12,42). The following model of the combining site has been derived from various discussions of observed combining site conformations (11-14,33,34,40-42). The positioning of hypervariable loops which form the antibody combining site is such that the L3 and H3 loops face each other, forming the center of the combining site. L1 residues 26-27b extend across the surface of the VL framework (27b side chain is buried within the framework of VL) while residues 27c-31 (variable in length) form a loop extending away from the surface; residues 32-34 descend into the combining pocket. L2 forms a 3-residue hairpin turn whose contribution to hapten binding may depend on the size of neighboring loops (33). H1 residues 26-33 extend across the VH framework and descend into the binding pocket. H2 residues 51-58 ascend from the combining pocket, form a loop structure of varying length and residues 59-65 continue down one side of the VH domain.

In attempting to identify potential antigen-contact residues, an assumption was made that the orientation of hapten bound by the combining site be similar to what may be encountered with the hapten-carrier immunogen; that is, the free end of the hapten (i.e. the choline in PC and the t-butyl moiety in Soman) would point into the combining site with the linked phenyl ring situated closer to the surface of the combining site. This is consistent with the observed orientation of PC hapten when bound by M603 (33-35).

In order to perform this analysis, we relied on what is known about the binding characteristics of these mAbs (4-7,43,44) and antigen-antibody interaction in general (40,45). Fine specificity studies of these anti-PC-KLH and anti-So-KLH mAbs determined that the phenyl moiety was recognized as a dominant feature (4,5,7,43,44,46,47). Several positions common to both sets of mAbs can be identified at which aromatic residues capable of interacting with the phenyl ring are conserved and which could approach the bound hapten. These include L1 residues 27d and 32, and H1 residues 32 and 33 (Figure 4a-d). L3 residue 96 is also predominantly aromatic and could interact with the phenyl-haptens from below, although the side chain of L96 is thought to be partially buried in the VL-VH interface (11). A number of aromatic residues also occur in the H3 loop, particularly among several anti-PC-KLH mAbs where 2 aromatic pairs are separated by 1 to 3 residues (Figure 4d). Interestingly, in the recently determined structure of Fab R19.9 (41), derived from a mAb specific for the aromatic hapten p-azobenzenearsonate, the side chains from a total of 9 aromatic residues contributed by both VH and VL CDRs were found oriented towards the solvent and thus could possibly participate as antigen contact residues.

A major difference between the Soman and PC haptens is the presence of a positively charged nitrogen in the choline moiety of PC whereas the homologous t-butyl moiety in Soman is hydrophobic (4,5). Fine specificity studies showed that the positively charged nitrogen is required for binding by these 8 anti-PC-KLH mAbs (7,44). Similar studies showed that the presence of this positive charge is detrimental to binding by anti-So-KLH antibodies (4,5). We looked for charge-complimentary residues capable of interacting with the choline nitrogen in anti-PC-KLH mAbs but which were absent or not conserved in anti-So-KLH mAbs. Two such positions were identified: L1 residue 34 and residue 58 (Figure 4a-d). Although residue 58 is not a hypervariable residue according to Chothia and Lesk (12), Kabat et al. include residue 58 as part of the second complimentarity determining region of the heavy chain (21). In addition, the resolved crystal structure of M603 indicates that Glu58 of the heavy chain can participate as an antigen contact residue (35). Of the 3 anti-PC-KLH mAbs which did not possess acidic residues at either position 34 or 58, PCG1-14 and PCG1-2 share Glu93 (L3) while 52-1 possess Glu56 (H2); both positions are still within the combining site and therefore could possibly interact with PC. Among the anti-So-KLH mAbs, only 2 possess an acidic residue at any of these positions. Identification of residues potentially capable of interacting with the phosphate or phosphonate is more difficult since strongly basic positions were not well conserved and numerous residues with hydrogen bonding capabilities were identified in both sets of antibodies. However, slightly basic His residues were predominant at several Vk positions including 27d and 93 in anti-PC-KLH mAbs and 27d, 34, and 93 in anti-So-KLH mAbs (Figure 4a & b). In the combining site of M603, Arg52 of the heavy chain is thought to play a significant role in the binding of PC through interaction with the phosphate group (33, 35). Other M603 residues which may

interact with the phosphate group include Tyr33 and Lys54 of the heavy chain. In the anti-Soman-KLH and anti-PC-KLH antibodies, these residues are not conserved. Consistent with heterogeneous V gene usage, it appears that combining site structure of anti-So-KLH and anti-PC-KLH antibodies is equally heterogeneous. Nevertheless, as described above, there do appear to be common features which could contribute to their ability to bind their cognate haptens. The non-cross-reactive specificity of these antibodies is maintained, however, most likely through the charge, hydrogen bonding, and shape complimentarity present in their respective antigen-binding sites.

Surface variability:

Surface variability was calculated according to the method of Keiber-Emmons et al. (23) and plotted for 3 sets of antibodies (Figure 5): a) 9 anti-SoKLH mAbs, b) 8 anti-PC-KLH mAbs, and c) M603 plus 6 other pneumococcal polysaccharide-binding antibodies derived from the same VL and VH genes as M603 (48). Similar areas of high surface variability were detected in all sets of antibodies and correlated with hypervariable regions extending as hairpin loops from the V domain surface. Framework residues surrounding VL residues 41-42 and 80-81 as well as VH residues 38 and 73 also showed some variability consistent with other reports that these regions are surface exposed (12-14,23). Highest surface variability was associated with residues 52a-55 (H2), residues 96-100 (H3), and 28-31 (L1). According to the resolved structure of M603(33-35), these particular regions do not make contact with the bound PC hapten, consistent with their positioning at the outer edges of the combining site loop. These residues are more likely to contribute to interaction with carrier determinants of the immunogen and/or to serve as antigenic determinants (idiotopes) themselves. Claflin et al. have suggested

that variability of residues lining the lip of the combining site may influence the ability of PC-binding proteins to recognize PC when it is associated with the surface structure of different organisms (49). A somatic variant of PC-binding myeloma S107 was isolated and found to have significantly decreased binding for PC-protein while retaining total binding for PC hapten (50). Interestingly, the only change detected was Asp to Ala at position 101 of the heavy chain; Asp101 forms a salt bridge with Arg 94 and influences the conformation of the H3 loop in M603 (12) and is thought to do so in S107 as well (36). If the H3 loop is important for carrier contact, disruption of the H3-associated salt bridge would explain the binding phenotype observed for this S107 mutant.

Despite the speculative nature of the modeling correlations presented, they offer plausible distinctions between antibodies to the two organophosphorus structures; as such they provide a basis for more detailed modeling as well as for direct experimentation through the application of molecular cloning and site directed mutagenesis.

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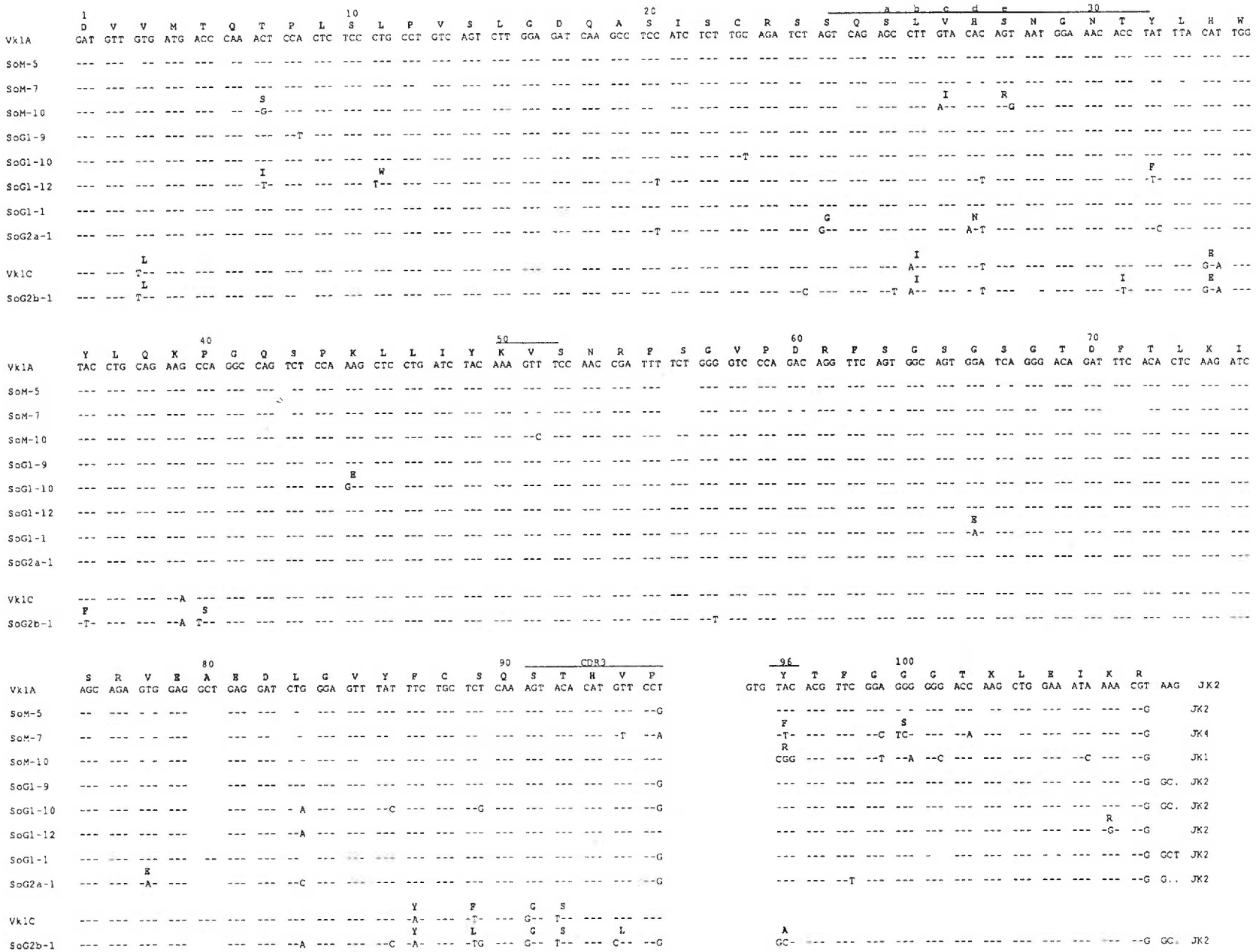
Table I: R:S mutations in Vk1 light chains

	SoG1-9	SoG1-10	SoG1-12	SoG1-1	SoG2a-1	SoG2b-1		
FR1	0:1	0:1	1:2	0:0	0:1	0:0		
FR2	0:0	1:0	0:0	0:0	0:0	2:0		
FR3	0:0	0:2	0:1	1:0	1:1	0:3		
FR4	0:1	0:1	1:1	0:1	0:2	0:1		
all FRs	0:2	1:4	2:4	1:1	1:4	2:4	=	7:19
CDR1	0:0	0:0	1:1	0:0	2:1	1:2		
CDR2	0:0	0:0	0:0	0:0	0:0	0:0		
CDR3	0:1	0:2	0:0	0:1	0:1	4:1		
all CDRs	0:1	0:2	1:1	0:1	2:2	5:3	=	8:10
FR + CDR	0:3	1:6	3:5	1:2	3:6	7:7	=	15:29

Figure 1: Vk sequences of 9 anti-So-KLH monoclonal antibodies.

Germline sequences for Vk1A and Vk1C were obtained from ref. 20.



	1									10										20								a	b	c	d	e			30							
	D	V	V	M	T	Q	T	P	L	S	L	P	V	S	L	G	D	Q	A	S	I	S	C	R	S	S	Q	S	L	V	H	S	N	G	N	T	Y	L	H	W
Vk1A	GAT	GTT	GTG	ATG	ACC	CAA	ACT	CCA	CTC	TCC	CTG	CCT	GTC	AGT	CTT	GGA	GAT	CAA	GCC	TCC	ATC	TCT	TGC	AGA	TCT	AGT	CAG	AGC	CTT	GTA	CAC	AGT	AAT	GGA	AAC	ACC	TAT	TTA	CAT	TGG
SoM-5	---	---	--	---	---	--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoM-7	---	---	---	---	---	---	---	---	---	---	---	--	---	---	---	---	---	---	---	---	---	---	---	---	---	--	---	---	---	-	-	---	---	---	---	---	---	-	---	---
SoM-10	---	---	---	---	---	--	S -G-	---	---	---	---	---	---	---	---	---	---	---	---	--	---	---	---	---	---	---	---	---	I A--	---	R --G	---	---	---	---	---	---	---	---	---
SoG1-9	---	---	---	---	---	---	---	--T	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG1-10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG1-12	---	---	---	---	---	---	I -T-	---	---	---	W T--	---	---	---	---	---	---	---	---	--T	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---	F -T-	---	---	---
SoG1-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG2a-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---	G G--	---	---	---	---	N A-T	---	---	---	---	---	--C	---	---	---
Vk1C	---	---	L T--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	I A--	---	--T	---	---	---	---	---	---	---	E G-A	---
SoG2b-1	---	---	L T--	---	---	---	---	--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--C	---	---	--T	I A--	---	- T	---	-	---	---	---	I -T-	---	E G-A	---

	Y	L	Q	K	40 P	G	Q	S	P	K	L	L	I	Y	50 K	V	S	N	R	F	S	G	V	P	60 D	R	F	S	G	S	G	S	G	T	70 D	F	T	L	K	I
Vk1A	TAC	CTG	CAG	AAG	CCA	GGC	CAG	TCT	CCA	AAG	CTC	CTG	ATC	TAC	AAA	GTT	TCC	AAC	CGA	TTT	TCT	GGG	GTC	CCA	GAC	AGG	TTC	AGT	GGC	AGT	GGA	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC
SoM-5	---	---	---	---	---	---	---	--	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoM-7	---	---	---	---	---	---	---	--	---	---	---	---	---	---	- -	---	---	---	---	---		---	---	---	- -	---	- -	- -	---	---	---	---	---	---	---		--	---	---	---
SoM-10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--C	---	---	---	---	---	--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG1-9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG1-10	---	---	---	---	---	---	---	---	---	E G--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG1-12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG1-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	E -A-	---	---	---	---	---	---	---	---	---
SoG2a-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Vk1C	---	---	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SoG2b-1	F -T-	---	---	--A	S T--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

	S	R	V	E	80 A	E	D	L	G	V	Y	F	C	S	90 Q	S	T	H	V	P
Vk1A	AGC	AGA	GTG	GAG	GCT	GAG	GAT	CTG	GGA	GTT	TAT	TTC	TGC	TCT	CAA	AGT	ACA	CAT	GTT	CCT
SoM-5	--	---	- -	---		---	---	-	---	---	---	---	---	---	---	---	---	---	---	--G
SoM-7	--	---	- -	---		---	--	-	---	---	--	---	---	--	---	---	---	---	-T	--A
SoM-10	---	---	- -	---		---	---	- -	---	--	---	---	---	--	---	---	---	---	---	---
SoG1-9	---	---	---	---		---	---	---	---	---	---	---	---	---	---	---	---	---	---	--G
SoG1-10	---	---	---	---		---	---	- A	---	---	--C	---	---	--G	---	---	---	---	---	--G
SoG1-12	---	---	---	---		---	---	--A	---	---	---	---	---	---	---	---	---	---	---	---
SoG1-1	---	---	---	---	--	---	---	--	---	---	---	---	---	---	---	---	---	---	---	--G
SoG2a-1	---	---	E -A-	---		---	---	--C	---	---	---	---	---	---	---	---	---	---	---	--G
Vk1C	---	---	---	---	---	---	---	---	---	---	---	Y -A-	---	F -T-	---	G G--	S T--	---	---	---
SoG2b-1	---	---	---	---		---	---	--A	---	---	--C	Y -A-	---	L -TG	---	G G--	S T--	---	L C--	--G

	96 Y	T	F	G	100 G	G	G	T	K	L	E	I	K	R		
	GTG	TAC	ACG	TTC	GGA	GGG	GGG	ACC	AAG	CTG	GAA	ATA	AAA	CGT	AAG	JK2
SoM-5		---	---	---	---	- -	---	---	--	---	---	---	---	--G		JK2
SoM-7		F -T-	---	---	--C	S TC-	---	--A	---	--	---	---	---	--G		JK4
SoM-10		R CGG	---	---	--T	--A	--C	---	---	---	---	--C	---	--G		JK1
SoG1-9		---	---	---	---	---	---	---	---	---	---	---	---	--G	GC.	JK2
SoG1-10		---	---	---	---	---	---	---	---	---	---	---	---	--G	GC.	JK2
SoG1-12		---	---	---	---	---	---	---	---	---	---	---	R -G-	--G		JK2
SoG1-1		---	---	---	---	-	---	---	---	---	- -	---	---	--G	GCT	JK2
SoG2a-1		---	---	--T	---	---	---	---	---	---	---	---	---	--G	G..	JK2
Vk1C																
SoG2b-1		A GC-	---	---	---	---	---	---	---	---	---	---	---	--G	GC.	JK2

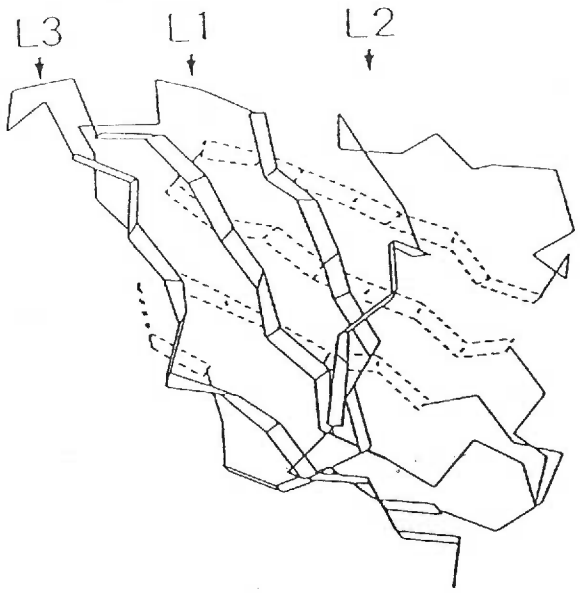
Figure 2: VH sequences of 9 anti-So-KLH monoclonal antibodies. Hybridoma SoG2b-1 contains a 3 bp deletion (dots) affecting codons 53-54. 19.1.2 VH is a member of the J558 family derived from an α1,6 dextran-specific hybridoma (29). TF2-76 VH is a member of the 7183 family derived from a DNP-specific hybridoma (28).

```
           1                                   10                                      20                                      30                                      40
           Q   V   Q   L   Q   Q   S   G   A   E   L   M   K   P   G   A   S   V   K   I   S   C   K   A   T   G   Y   T   F   S   S   Y   W   I   E   W   V   K   Q   R      VH family
19.1.2    CAG GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG ATG AAG CCT GGG GCC TCA GTG AAG ATA TCC TGC AAG GCT ACT GGC TAC ACA TTC AGT AGC TAC TGG ATA GAG TGG GTA AAG CAG AGG     J558
                                                                                               S
SoM-10    --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---     J558
                                                                                                           R                                               E
SoG1-10   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -G- --- --- --- --T --- --- --- --- --- --- G-- ---     J558
           E                                       V                                                   S       S       T   G       Y   M   H                   S
SoM-5     G-- --C --- --- --- --- --- --- C-  --C --- G-- --- --- --- --T --- --- --- --- --- --- --- --- T-- --T --- T-- --- -C- G-- --- -AC --G C-C --- --G --- --- --C     J558
           E                                       V           R   T       M                               S       S       T   G       T       H       M           S
SoG2a-1   G-- --C --- --- --A --- --- --- C-  --- --- G-- --- --- A-A A-T --- A-- --- --- --- --- --- --- T-- --T --- T-- --- -C- G-- --- ACC --- C-C --- A-G --- --- --C     J558
           E                                       V                                                   T   S                           E       T   M   H                   S
SoG1-9    G-- --C --- --- --A --- --- --- C-  --- --- G-- --- --- --- --T --- --- --- --- --- --- --- A-- T-- --A --- --- --- --- GAA --- ACC --G C-C --- --G --- --- --C     J558
                                                   A                               M                       S               T                   M   H
SoM-7     --- --C --- --T --- --- --- --- --- --A --- GCA -A  --- --- --- --- --- --- --G --- --- --- --- T-- --- --- --C --T -C- --- --- --- --G C-C --- --- --A --- ---     J558
                       M                           V                               L               T   S       F                   S   Y       S       L           K
SoG1-12   --- --- --- A-- --- --- --- --- --- --- --T G-- --- --- --- --T --- --- --A C-G --- --- --- A-- T-- --- -T- --C --- --C --T AGT -AT --- AGT --- T-G --- --A -A-     J558
                                   P               V                               L                       S               T           L   M   H
SoG1-1    --- --C --A --- --- --- C-- --- --- --A --- G-- --- --- --- --T --- --- --- C-G --- --- --- --- T-- --- --- --C --- -CC --T --- -T- --G C-C --- --G --- --- ---     J558

           E   V   M   L   V   E   S   G   G   G   L   V   K   P   G   G   S   L   K   L   S   C   A   A   S   G   F   T   F   S   S   Y   A   M   S   W   V   R   Q   T
TF2-76    GAA GTG ATG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TAT GCC ATG TCT TGG GTT CGC CAG ACT     7183
                                                                                                               T                   T       S
SoG2b-1                                       T-  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ACT --- --- --- -C- --- T-- --- --- --- --- --- --- ---     7183


                                               50          52a D   G                   60                                      70
           P   G   H   G   L   E   W   I   G   E   I   L   P   G   S   G   S   T   N   Y   N   E   K   F   K   G   K   A   T   F   T   A   D   T   S   S   N   T   A   Y
19.1.2    CCT GGA CAT GGC CTT GAG TGG ATT GGA GAG ATT TTA CCT GGA AGT GGT AGT ACT AAC TAC AAT GAG AAG TTC AAG GGC AAG GCC ACA TTC ACT GCA GAT ACA TCC TCC AAC ACA GCC TAC
                                                                   R                       T
SoM-10    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                                                                                   P
SoG1-10   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- ---
           H       K   S                       R   V   N       N   N       G       S           Q                           I   L       V       K           S
SoM-5     -A- --- A-G A-- --- --- --- --- --- CGT G-- AAT --- AAC -A- --- G-- --- -G- --- --C C-- --- --- --- --- --- --- -T- --A --- -T- --C -AG --A --- -G- --- --- ---
           H       K   N                       L       N       Y   N       G       T           Q                               L       V       K           S
SoG2a-1   -A- --- A-G AA- --- --- --- --- --- CTT --- AAT --- TAC -A- --- G-- --- -C- --- --C C-- --- --- --- --- --- --- --- --A --- -TT --C -AG --A --- -G- --- --- ---
           H       K   S                       G       N       K   N       G       S           Q   N                       S   L       V       K           S
SoG1-9    -A- --- A-G A-- --- --- --- --- --- -GT --- AAT --- AA- -A- --- G-- --- -GT --- --C C-- --C --- --- --- --- --- T-- --G --- -T- --C -AG --- --- -G- --- --- ---
                   Q                           Y       N       S   T       Y       E           Q               D               L               K           S
SoM-7     --- --- --G --T --G --A --- --- --- T-C --- AAT --- A-C -C- --- TA- --- G-G --- --- C-- --- --- --- -A- --- --- --- --G --- --- --C -A- --- --- -G- --- --- ---
                   Q   S                   A   W       Y   A       T       V       S           Q           T                   L       V           P       S
SoG1-12   --- --- --G A-T --- --- --- --- -C- TG- --- -AT G-- --- -C- --- GT- --- -GT --T --- C-- --- --- -CA --- --- --- A-  C-G --- -T- --C --G C-- --- -G- --- --- ---
                   Q                                   N       S   N       R                                   N               L       V       K   P       S
SoG1-1    --- --- --A --- --- --- --- --- --- --- --- AAT --- A-C -AC --- C-- --- --- --- --- --A --A --T --- AA- --- --- --- C-G --- -T- --C -A- C-- --- -G- --- --- ---

           P   E   K   R   L   E   W   V   A   T   I   S   S   G   G   T   Y   T   Y   Y   P   D   S   V   K   G   R   F   T   I   S   R   D   N   A   K   N   T   L   Y
TF2-76    CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT ACT TAC ACC TAC TAT CCA GAC AGT GTG AAG GGG CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC
               D                               S   L   T               S                                                                               R       I
SoG2b-1   --A --C --- --- --- --- --- --- --- T-- C-- -C- --- --- --- -G  --- --- --- --- --- --- --- --- --- --C --- --- --- --- --- --- --T --C --- -G- --- -T- --- ---


           80      82a 82b 82c                     90                                      D region                                  101        J region       110
           M   Q   L   S   S   L   T   S   E   D   S   A   V   Y   Y   C   A   R                                                      Y   F   D   Y   W   G   Q   G   T   T   L   T   V   S   S
19.1.2    ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCC GTC TAT TAC TGT GCA AGG                                                    TAC TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA   J2
                                                                               H    P   P   L   Y   Y   R   Y   D   G   Y
SoM-10    --- --G --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A CAC  CCC CCG CTC TAC TAT AGG TAC GAC GGG TAC    --- --- --- --- --- --- --- --- --- --- --- ---               J2
               E                                                                    G   E   S   G                                          D                           L   V                   A
SoG1-10   --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A      GGG GAA TCC GGA G..                                    ,-- --- --- --- --G --T CTG G-- --  - - --T G--   J3
               E       R                                                            D   Y   D   Y   D   R
SoM-5     --- G-G --- C-- --- --- --- --- --- --- --- --G --- --- --- --- --- --A      GAC TAT GAT TAC GAC AGG                            --  --- --- --- --- --- --- --- --,                   J2
               E       L                                                            E   G   G   N   Y   G                                                          L   V                   A
SoG2a-1   --- G-G --- CT- --T --- --- --- --- --- --- --A --- --- --- --- --- --A      GAG GGG GGt AAT TAC GGG                                    ---  -  --- --G  -T CTG G-- --T --- --T G--   J3
               E       R                                                   K        W   G   G                                                                              A
SoG1-9    - - G-G --- C-- --- --- --- --- --- --- --T --A --- --- --- --- --- -AA      TGG GGT GGC                                        --- --- --- --- --- --- --- --- --- --- -C-           J2
                                                                                    N   G   Y   G
SoM-7     --- --- --G --- --- --- --- --- --- --- --- --A - - --- --- --- --- --A      AAC GGC TAC GG.                                    --- --- --- --- --  --- --- --- --,
                   F                   T   Q           I                       H    Y   Y                                                      N                   P   L   V
SoG1-12   --- --- T-- --- --- --- --- A-- C-- --- --- --- A-- --- --- --- --- --A CAC  TAC TAT                                            --T A-- --- --- --- --G C-T CTG G-- --T ---           J3
                                                                       V       H    S   G   P
SoG1-1    --- --- --- --- --- --- --- --- --- --- --- --G --- --- --- --- -T- --A CAC  TCT GGA CC.                                        --- --- --- --T --- --- --- --- --- --- --- --- ---   J2

           L   Q   M   S   S   L   R   S   E   D   T   A   M   Y   Y   C   A   R
TF2-76    CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC ACG GCC ATG TAT TAC TGT GCA AGA
                                                                                    G   R   W   G   N   Y   A   F              A                           L   V
SoG2b-1   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---      GGC CGA TGG GGT AAC TAC GCC TTT            -CT --- --- --- --- --G --T CTA G-- --T ---           J3
```

Figure 3a: Structure of an immunoglobulin VL domain. Strands of β-sheet are represented by ribbons with the dashed line ribbons representing one β-sheet and the solid line ribbons representing the other β-sheet. The domain is viewed from the β-sheet that forms the VH-VL interface. (Adopted from C. Chothia and A. M. Lesk (12).)

Figure 3b: Plane of the β-sheet framework conserved in the VH and VL domains of known atomic structure. In VL, the top half of the plane corresponds to the dashed line β-sheet in Figure 3a and the bottom half of the plane corresponds to the solid line β-sheet. Jagged lines correspond to hypervariable loops and dashed lines represent hydrogen bonds formed between main chain atoms of β-strands within a β-sheet. (Adopted from C. Chothia and A. M. Lesk (12).)

a)


L3
L1
L2

b)

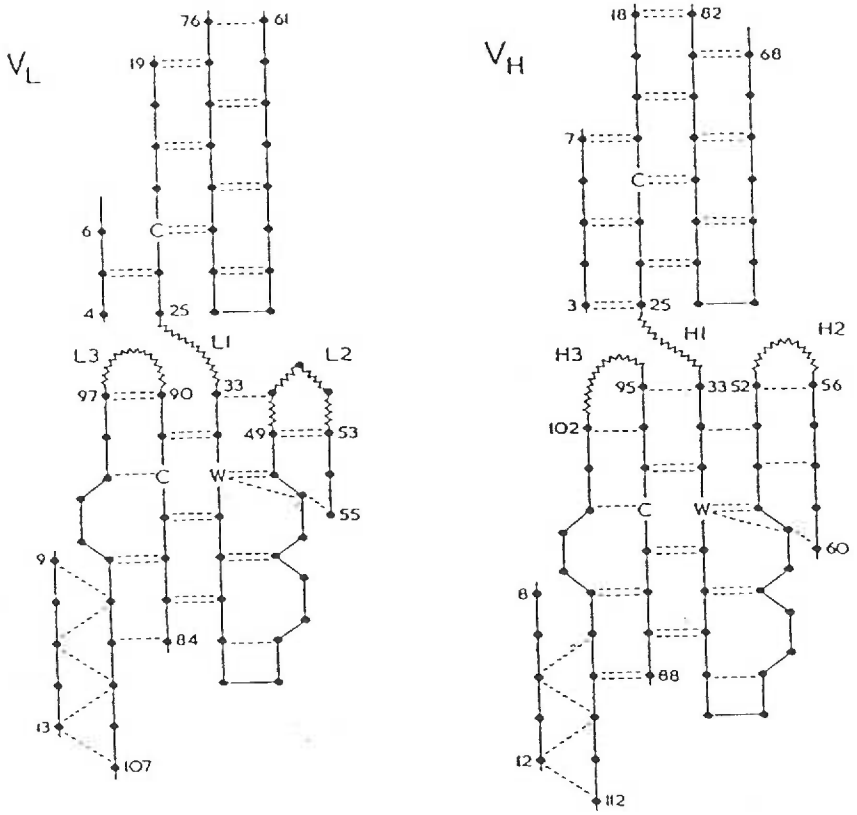
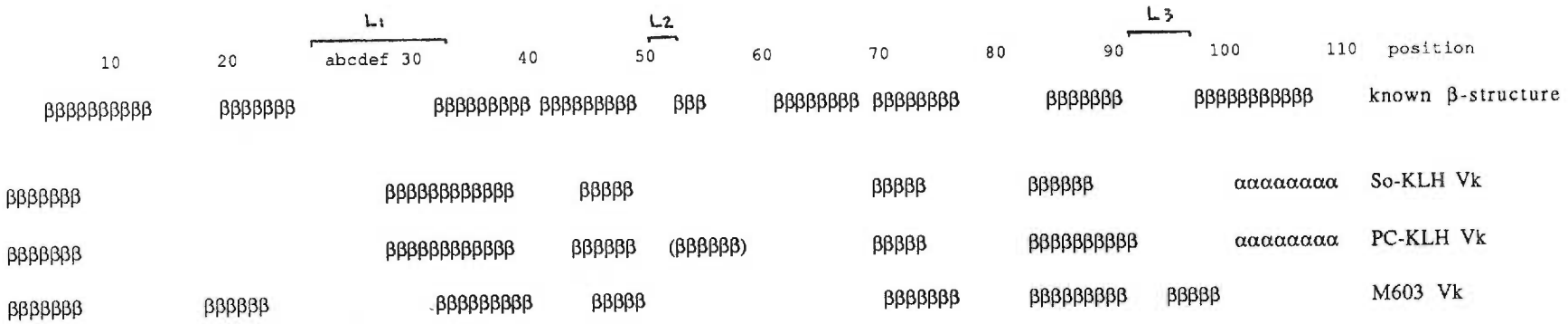

V_L
76
61
19
6
C
4
25
L3
L1
L2
97
90
33
49
53
C
W
55
9
84
13
107
V_H
18
82
68
7
C
3
25
H1
H2
H3
95
33
52
56
102
C
W
60
8
88
12
112

Figure 3c: Secondary structure predicted for 9 anti-So-KLH VH andVk sequences, 8 anti-PC-KLH VH and Vk sequences and M603 VH and Vk. β-structure in parentheses were found in most but not all sequences. βββ = beta strand; ααα = alpha helix. Regions which form neither beta strand nor alpha helix may form beta turns or do not have well-ordered secondary structure.

Secondary structure predicted for Vk sequences:



Secondary structure predicted for VH sequences:

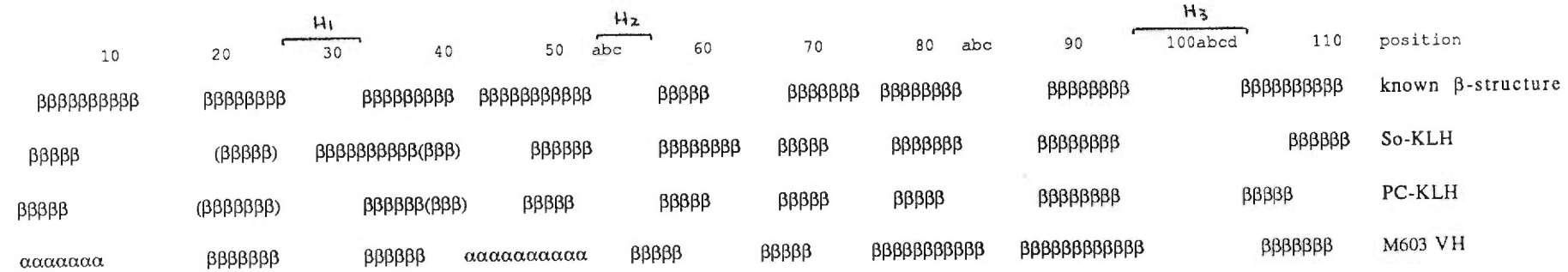


Figure 4 a) Light chain L1 and L2 hypervariable regions of anti-So-KLH and anti-PC-KLH mAbs. Boxed regions designate hypervariable regions according to Chothia and Lesk (12). Phe32 is known to contact bound PC in the known structure of M603 (refs. 33-35) and is outlined in the M603 Vk sequence.

clone	25	26	27	a	b	c	d	e	f	28	29	30	31	32	33	34	50	51	52
anti-PC-KLH:								L1										L2	
104-8	S	S	Q	R	I	V	H	S		N	G	N	T	Y	L	E	K	V	S
111-1	S	S	Q	S	I	V	H	S		N	G	N	T	Y	L	E	K	V	S
PCG2a-1	S	S	Q	S	I	V	H	S		N	G	N	T	Y	L	E	K	V	S
PCG1-1	S	S	Q	S	I	V	H	S		N	G	N	T	Y	L	E	K	V	S
PCG1-14	S	S	K	S	L	L	Y	K		D	G	K	T	Y	L	N	L	M	S
PCG1-2	S	T	K	S	L	L	Y	K		D	G	K	T	Y	L	N	L	M	S
52-1	S	S	Q	S	L	V	I	S		N	G	N	T	Y	L	H	K	V	S
PCG2b-2	S	S	Q	S	I	V	Y	G		D	G	N	T	Y	L	E	E	V	S
M603	S	S	Q	S	L	L	N	S	G	N	Q	K	N	F	L	A	G	A	S
anti-So-KLH:																			
SoM-5	S	S	Q	S	L	V	H	S		N	G	N	T	Y	L	H	K	V	S
SoM-7	S	S	Q	S	L	V	H	S		N	G	N	T	Y	L	H	K	V	S
SoM-10	S	S	Q	S	L	V	H	R		N	G	N	T	Y	L	H	K	V	S
SoG2b-1	S	S	Q	S	I	V	H	S		N	G	N	T	Y	L	E	K	V	S
SoG1-1	S	S	Q	S	L	V	H	S		N	G	N	T	Y	L	H	K	V	S
SoG1-9	S	S	Q	S	L	V	H	S		N	G	N	T	Y	L	H	K	V	S
SoG1-10	S	S	Q	S	L	V	H	S		N	G	N	T	Y	L	H	K	V	S
SoG1-12	S	S	Q	S	L	V	H	S		N	G	N	T	F	L	H	K	V	S
SoG2a-1	S	G	Q	S	L	V	N	S		N	G	N	T	Y	L	H	K	V	S

Figure 4 b) L3 hypervariable regions of anti-So-KLH and anti-PC-KLH mAbs. Boxed regions designate hypervariable regions according to Chothia and Lesk (12). M603 residues Asp91, His92, Tyr94, and Leu96 are known to contact bound PC (refs. 33-35) and are outlined. * Position 96 in hybridoma 52-1 remains undetermined and may be Phe, Ile, or Val.

clone anti-PC-KLH:	89	90	91	92	93	94	95	96	97	
					L3					
104-8	F	Q	G	S	H	V	P	W		T
111-1	F	Q	G	S	H	V	P	W		T
PCG2a-1	F	Q	G	S	H	V	P	W		T
PCG1-1	F	Q	G	S	H	F	P	Y		T
PCG1-14	Q	Q	L	V	E	Y	P	F		T
PCG1-2	Q	Q	L	V	E	Y	P	F		T
52-1	S	Q	S	T	H	V	P	*		T
PC2b-2	F	Q	G	S	H	V	P	Y		T
M603	Q	N	D	H	S	Y	P	L		T

anti-So-KLH:	89	90	91	92	93	94	95	96	97	
SoM-5	S	Q	S	T	H	V	P	Y		T
SoM-7	S	Q	S	T	H	V	P	F		T
SoM-10	S	Q	S	T	H	V	P	R		T
SoG2b-1	L	Q	G	S	H	L	P	A		T
SoG1-1	S	Q	S	T	H	V	P	Y		T
SoG1-9	S	Q	S	T	H	V	P	Y		T
SoG1-10	S	Q	S	T	H	V	P	Y		T
SoG1-12	S	Q	S	T	H	V	P	Y		T
SoG2a-1	S	Q	S	T	H	V	P	Y		T

Figure 4 c) Heavy chain H1 and H2 hypervariable regions of anti-So-KLH and anti-PC-KLH mAbs. Boxed regions designate hypervariable regions according to Chothia and Lesk (12). M603 residues Tyr33, Arg52, Asn53, Lys54, and Glu58 are known to contact bound PC (refs. 33-35) and are outlined.

H1 sequences in parentheses assigned to PCG2b-2 are those of a homologous 7183 family member (8) since the sequences corresponding to this region of PCG2b-1 have not been determined.

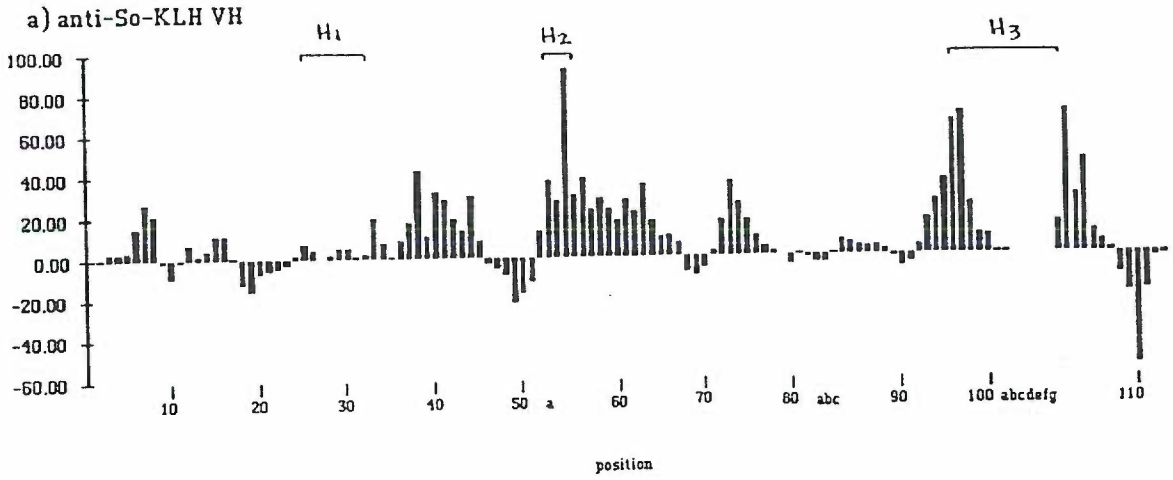
clone	26	27	28	29	30	31	32	33	34	51	52	a	b	c	53	54	55	56	57	58
anti-PC-KLH:				H1											H2					
104-8	G	F	T	F	S	N	F	Y	M	S	R	N	K	A	Q	D	Y	T	T	E
111-1	G	F	T	F	S	D	F	Y	M	S	R	N	K	A	Y	G	Y	T	T	E
PCG2a-1	G	F	T	F	T	D	Y	Y	M	I	R	N	K	A	F	H	Y	T	T	E
PCG1-1	G	F	S	L	T	V	Y	G	V	I	W				G	D	G	S	T	D
PCG1-14	G	Y	T	F	T	E	Y	I	I	F	Y	P			G	S	G	Y	I	K
PCG1-2	G	Y	T	F	T	E	Y	I	L	F	Y	P			G	S	G	Y	I	K
52-1	G	X	T	F	T	N	C	G	M	I	N	T			N	T	G	E	P	T
PCG2b-2	(G	F	T	F	S	S	Y	T	M)	I	S	N			G	G	G	R	I	Y
M603	G	F	T	F	S	D	F	Y	M	S	R	N	K	G	N	K	Y	T	T	E
anti-So-KLH:																				
SoM-5	G	Y	S	F	T	G	Y	Y	M	V	N	P			N	N	G	G	T	S
SoM-7	G	Y	T	F	T	S	Y	W	M	I	N	P			S	T	G	Y	T	E
SoM-10	G	Y	T	F	S	S	Y	W	I	I	L	P			G	R	G	S	T	N
SoG2b-1	G	T	T	F	S	T	Y	S	M	L	T				S	G	G		S	T
SoG1-1	G	Y	T	F	S	S	Y	L	M	I	N	P			S	N	G	R	T	N
SoG1-9	G	Y	T	F	S	E	Y	N	M	I	N	P			N	K	G	G	T	S
SoG1-10	G	Y	R	F	S	S	Y	W	I	I	L	P			G	S	G	S	T	N
SoG1-12	G	F	T	F	S	S	S	Y	I	I	Y	A			G	T	G	V	T	S
SoG2a-1	G	Y	S	F	F	T	G	Y	I	I	N	P			Y	N	G	G	T	T

Figure 4 d) Heavy chain H3 hypervariable regions of anti-So-KLH and anti-PC-KLH mAbs. Boxed regions designate hypervariable regions according to Chothia and Lesk (12). M603 residues Asn95 and Trp100a are known to contact bound PC (refs. 33-35) and are outlined. Due to the variable length of the H3 loop, the numbering system of Kabat et al. (21) assigns letters (i. e. a, b, c) after position 100 in order to maintain consistent numbering of framework 4 residues. ** Position 100 in H3 of PCG2a-1 remains undetermined and may be Phe or Leu.

clone	94	H3	103
anti-PC-KLH:	|		|
104-8	R G	H Y D Y F D	F W
111-1	R G	Y Y D Y F D	V W
PCG2a-1	R G	Y Y S T ** F D	Y W
PCG1-1	R A	Y Y R Y D W F A	Y W
PCG1-14	R H	E D G G Y W V A	Y W
PCG1-2	R H	G T D W G F A	Y W
52-1	R P	Y Y G S T G X T	Y W
PC2Gb-2	R G	G S Y Y G Y Y G L D	Y W
M603	R N	Y Y G S T W Y F D	V W
		100 a b c	

	94	H3	103
anti-So-KLH:	|		|
SoM-5	R D	Y D Y D R X	Y W
SoM-7	R N	G Y G	Y W
SoM-10	R H	P P L Y Y R Y D G Y Y F D	Y W
SoG2b-1	R G	R W G N Y A F A	Y W
SoG1-1	R H	S G P	Y W
SoG1-9	K W	G G D	Y W
SoG1-10	R G	E S G	D W
SoG1-12	R H	Y Y D	N W
SoG2a-1	R E	G G N Y	G W

Figure 5 a) Surface variability plot of 9 anti-So-KLH VH (a) and VL (b). Surface variability plots were generated using the method described by Kiebber-Emmons et al. (23) and is calculated as the product of variability in hydropathicity at a given amino acid position and the average hydropathicity at that position.


a) anti-So-KLH VH
H1
H2
H3
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
-60.00
10
20
30
40
50
a
60
70
80
abc
90
100
abcdefg
110
position

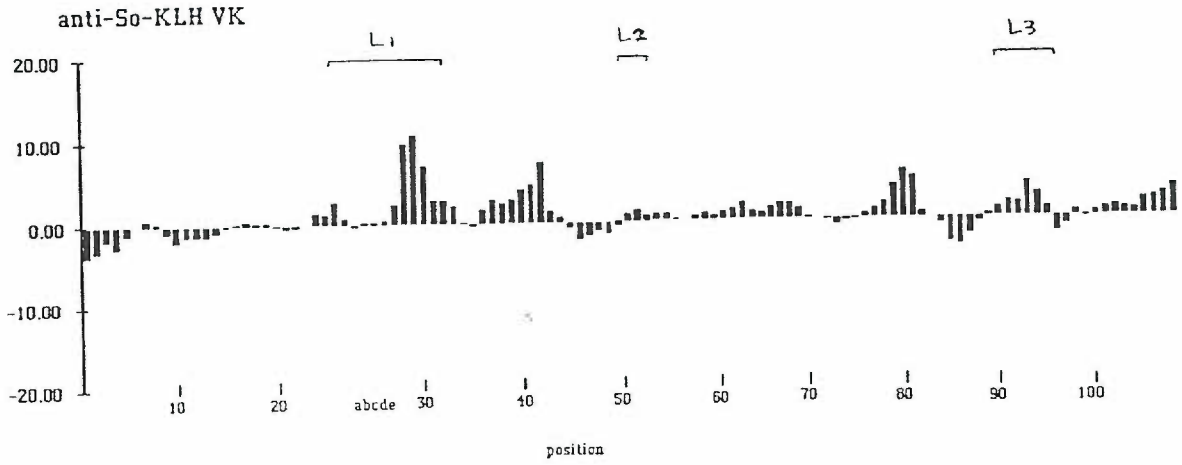
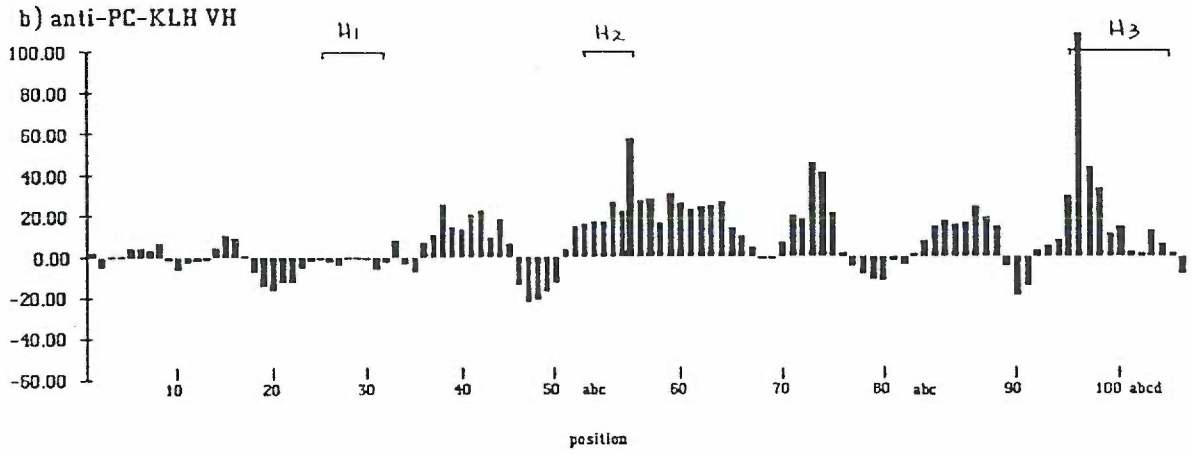

anti-So-KLH VK
L1
L2
L3
20.00
10.00
0.00
-10.00
-20.00
10
20
abcde
30
40
50
60
70
80
90
100
position

Figure 5 b) Surface variability plot of 8 anti-PC-KLH VH (a) and VL (b). Surface variability plots were generated using the method described by Kiebber-Emmons et al. (23) and is calculated as the product of variability in hydropathicity at a given amino acid position and the average hydropathicity at that position.


b) anti-PC-KLH VH
H1
H2
H3
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
-60.00
10
20
30
40
50
abc
60
70
80
abc
90
100
abcd
position

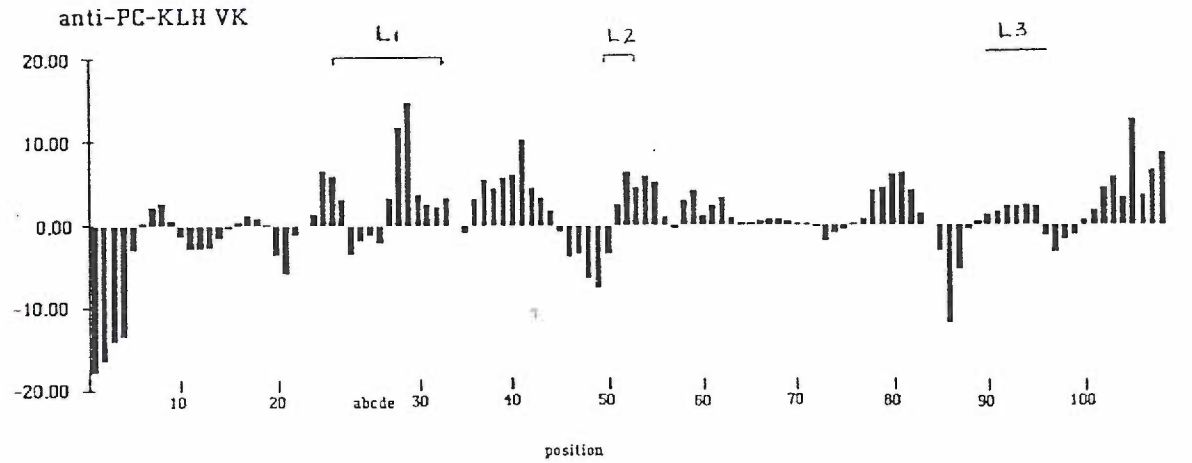
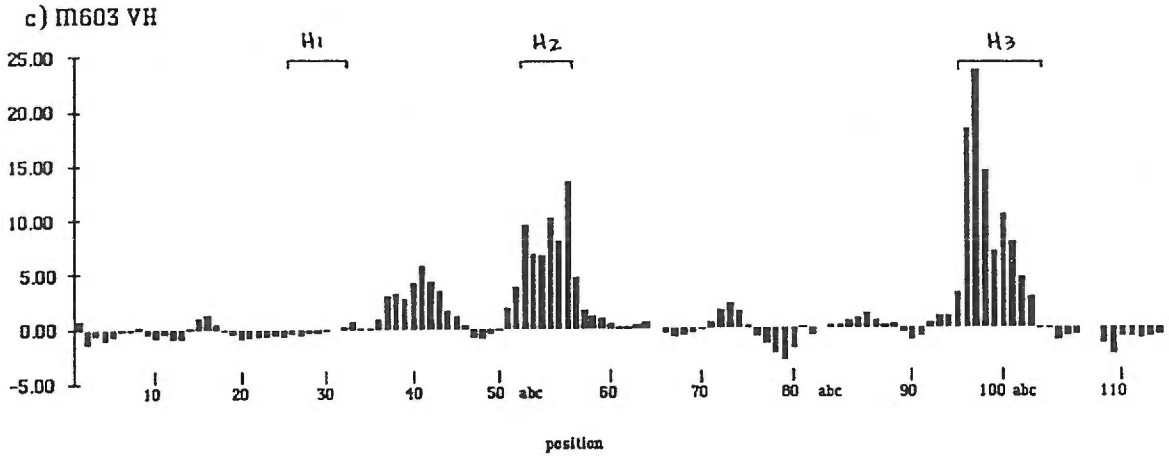
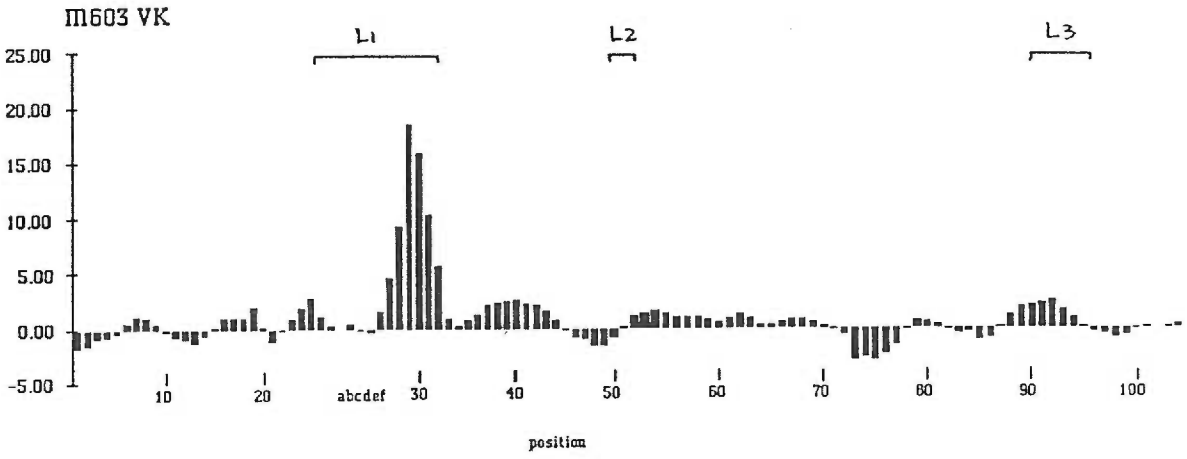

anti-PC-KLH VK
L1
L2
L3
20.00
10.00
0.00
-10.00
-20.00
10
20
abcde
30
40
50
60
70
80
90
100
position

Figure 5 c) Surface variability plot of VH (a) and VL (b) from M603 and M603-like proteins (48). Surface variability plots were generated using the method described by Kiebber-Emmons et al. (23) and is calculated as the product of variability in hydropathicity at a given amino acid position and the average hydropathicity at that position.


c) M603 VH
H1
H2
H3
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
10
20
30
40
50 abc
60
70
80 abc
90
100 abc
110
position


M603 VK
L1
L2
L3
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
10
20
abcdef
30
40
50
60
70
80
90
100
position

Paper 4

Maturation of the Serum Antibody Response To Soman-KLH

Abigail C. Buenafe and Marvin B. Rittenberg

INTRODUCTION

Soman [O-1,2,2 trimethylpropylmethylphosphonofluoridate] is an organophosphorus-containing neurotoxin which binds to acetylcholinesterase (1). We have characterized 46 murine monoclonal antibodies against Soman-keyhole limpet hemocyanin (So-KLH) (2,3) in order to define the structural elements important in antibody recognition of the Soman-protein epitope which has structural features resembling those of phenyl-phosphocholine, another organophosphorus-containing hapten studied previously (4-7). We have reported that hybridomas generated at different times during the response to So-KLH display a large degree of heterogeneity in both fine structure recognition and V gene usage (4-5). We have not previously described the maturation of the serum antibody response to So-KLH which is the subject of this communication.

The mechanisms directing the maturational changes in antibody found in immune memory to hapten-protein conjugates are known to involve clonal recruitment, somatic mutation and antigen selection (7-10) but are not yet clearly understood at the level of the molecular interactions between antigen and antibody. We are interested in defining these interactions with organophosphorus containing ligands. The immune response to phosphocholine-coupled keyhole limpet hemocyanin (PC-KLH) is characterized by primary response antibodies which are highly restricted in V gene usage and fine specificity (7,11-13). We have shown that following secondary challenge there is a dramatic shift to a more heterogeneous pool of antibodies, a large proportion of which have a unique specificity and represent the products of many different V gene combinations (4-7,14,15). So-

KLH is similar to PC-KLH in hapten structure and carrier coupling but differs significantly in charge groups (4,5). We show here the temporal changes that occur in fine specificity and antigen binding of anti-Soman serum antibodies as the immune response to So-KLH proceeds. Our findings are consistent with ongoing selection and expansion of B cell clones specific for the immunogen and suggest that the protein carrier may influence the selection process. In general, the fine specificities found in the serum antibody population correlate well with those described for individual Soman-specific hybridomas (4,5) and are likely to be encoded by multiple V gene combinations.

MATERIALS and METHODS

Animals:

Female Balb/c mice were obtained from Jackson Laboratories, Bar Harbor, ME, and were first immunized at approximately six weeks of age.

Immunization and serum collection:

Mice were immunized as indicated in Figure 2 and bled from the periorbital venous sinus on the days reported. Serum samples were stored at -20°C.

Quantification of Soman-specific serum IgM, IgG1 and κ antibodies:

Serum antibodies were quantified by direct binding in an ELISA. Wells of ELISA microtiter plates were coated overnight at room temperature with 2 ug/ml So-BSA in carbonate-bicarbonate buffer, pH 9.6. Plates were blocked with PBS-1% BSA for one hour and washed with PBS-0.05% Tween 20. Serum samples diluted in PBS-1% BSA were added and incubated for 2 hrs. Alkaline phosphatase-labeled rabbit anti-mouse IgM, IgG1 or Igκ was added after washing and incubated for 2 hr. Isotype-specific reagents were passed over columns containing multiple mAbs of other isotypes in order to remove cross-reactivity and retained <2% cross-reactivity with other isotypes under the conditions used. The amount of IgM, IgG1 or Igκ present in each serum sample was determined from standard curves using a mixture containing known concentrations of affinity-purified anti-Soman monoclonal antibodies of various isotypes. Plates were washed and *p*-nitrophenyl phosphate (1mg/ml, Sigma, St. Louis, MO.) in 0.9 M diethanolamine containing 1mM $MgCl$, pH 9.8, was added. Absorbance was read at 405 nm after approximately 2 hr.

incubation at room temperature. Measurements were calculated at serum dilutions derived from the linear portion of a titration curve.

Soman analogs and hapten inhibitors:

Haptens used in fine specificity analysis included the following Soman derivatives. *p*-Aminophenyl-Soman (APSo), *p*-nitrophenyl-Soman (NPSo), dipinacolylmethylphosphonate (DPMP), and hydroxy-Soman (OH-So) were provided by Dr. D. Lenz (US-AMRICD, Aberdeen Proving Ground, MD.). Other inhibitors included *p*-nitrophenylphosphocholine (NPPC; Sigma, ST. Louis, MO.), PC_{10}-BSA, So_{35}-OVA, So_7-BSA and So_{596}-KLH; the synthesis of So-protein conjugates was described previously (2). *p*-Nitrophenyl-3,3-dimethylbutylphosphonate (NPDBP), *p*-nitrophenylmethylphosphocholine (NPMPC) and *p*-nitrophenylethylphosphate (NPEP) were synthesized by Drs. U. Bruderer and J. Fellman. Figure 1 illustrates the chemical structure of these compounds.

Fine Specificity Analysis:

The fine specificity of Soman-specific antibodies was determined in an inhibition ELISA as described previously (2). Percent inhibition was calculated using a standard curve set up with each assay. The I_{50} value for each inhibitor is expressed as the mM concentration required for 50% inhibition of anti-Soman antibody binding to So-BSA.

Hybridomas:

Soman-specific hybridomas were generated and monoclonal antibodies were affinity purified as described (2,3).

RESULTS and DISCUSSION

Kinetics of the serum antibody response to So-KLH:

Levels of Soman-specific IgM and IgG1 were measured in serum samples pooled from 13-15 mice at various timepoints after immunization with So-KLH in Complete Freunds adjuvant. As shown in Figure 2, the primary anti-So-KLH response consisted mainly of IgM antibodies. The peak IgM response, however, was recorded on day 21 after a secondary boost. On days 10 and 14 of the primary response, serum IgG1 antibody was approximately one third the level of IgM antibody. After secondary immunization using So-KLH in incomplete Freunds adjuvant, IgG1 levels increased rapidly (compare day 14 to day 21) and peaked at day 35 before decreasing gradually. Mice were rested for 1 year before memory challenge with So-KLH. At this point, the 11 remaining mice were divided into 3 groups : G124A (n=3) received 0.01 ug , G124B (n=4) received 0.1 ug, and G124C (n=4) received 1.0 ug of So-KLH in saline, intravenously. Figure 2 shows the difference in anti-Soman serum IgM and IgG1 levels in response to boosting with different amounts of So-KLH. The IgG1 memory response is both rapid and vigorous in 2 (G124B and G124C) of the 3 groups tested. The relatively poor response of G124A animals is most likely due to administration of a suboptimal dose of Soman-KLH. The development of significant levels (up to 4000 ug/ml) of Soman-specific IgG1 serum antibody by day 7 after memory boost (compare to primary response day 7 where the average response was < 10 ug/ml) indicates that efficient priming of the Soman-specific repertoire had taken place and that long term memory for Soman-KLH lasting for at least 1 year was established in these mice. In contrast, the levels and kinetics of the IgM response after memory boost do not

differ significantly from that seen in the primary response and presumably represents a fresh primary response. Fine specificity changes occurring in the Soman-specific B cell pool during memory development was investigated in the serum samples collected throughout this immunization period.

Fine specificity of serum IgM anti-Soman-KLH antibodies:

The fine specificity of Soman-specific serum IgM antibodies was analyzed by competitive inhibition ELISA. I_{50} values for each inhibitor measured during the course of the IgM response are given in Table I. Inhibition curves demonstrating the ability of day 21 IgM antibodies to bind to Soman-BSA-coated plates in the presence of various inhibitors are shown in Figure 3. It is clear from these data that free hapten forms of Soman were unable to inhibit IgM antibodies while protein-coupled forms of Soman served as efficient inhibitors. The lack of efficient inhibition by PC-BSA and KLH indicates that the IgM antibodies measured were indeed Soman-protein-specific. Previous studies have shown that Soman-specific monoclonal antibodies cross-react poorly with PC analogs (2,3). The ability of serum IgM antibodies to recognize only the Soman-protein form may be due to required contributions of the protein carriers. Alternatively it may be that univalent Soman hapten is far less efficient at blocking the interaction between a substrate with multiple Soman epitopes (i.e. So-BSA-coated wells) and multivalent anti-Soman IgM antibodies. This seems unlikely since the amounts of free hapten tested ranged up to 10^5-fold greater than the I_{50} concentrations for hapten administered as hapten-protein. Finally, IgM-producing B cell clones are often associated with the early response to protein antigens and it has been suggested that they often express germline V gene sequences (16-18). It is

therefore possible that the Soman-protein-specific IgM antibodies detected here are products of newly stimulated B cells which have not yet been driven to mutate and/or subjected to selective pressures and consequently have poor affinity for the antigen.

Fine specificity of serum IgG1 and IgG2a anti-Soman-KLH antibodies:

Figure 4 demonstrates the fine specificity profiles of day 35 IgG1 and IgG2a antibodies. I50 values for various inhibitors at timepoints other than day 35 are also given in Table I. In contrast to what was observed with Soman-protein-specific IgM antibodies, IgG1 and IgG2a serum antibodies were efficiently inhibited by Soman haptens. In terms of relative efficiency, however, protein-coupled forms of Soman are 10 to greater than 1000-fold better as inhibitors than the free hapten forms tested and is unlikely to be solely attributable to the high valency of Soman-protein conjugates. Rather, antibody recognition of hapten may be influenced significantly by the protein carrier. Consistent with this is the observation that the immunogen So-KLH (hapten density = 0.074/1000 MW protein) is 20-30 fold better as an inhibitor of Soman-specific monoclonal and polyclonal serum antibodies than So-BSA (hapten density = 0.1/1000 MW protein).

Among the haptens, inhibition appeared to correlate with structural homology to the immunogen, Soman-KLH. NPSo and APSo most closely resemble this immunizing form since both bear a phenyl group which represents part of the linkage formed when Soman is coupled to tyrosine or histidine residues of the protein carrier (see Figure 1). The hapten NPDBP also contains the phenyl linker group but possesses a negatively charged phosphate, has an additional carbon between the phosphate and the free end

and lacks the carbon-1 branched methyl group found in Soman analogs. As a consequence of these changes, NPDBP does not inhibit as well as APSo and NPSo. The Soman analog DPMP lacks the phenyl structure and is generally a less efficient inhibitor than NPDBP. NPPC is structurally similar to NPDBP except that NPPC has a positively charged nitrogen in place of carbon 3 in the tertiary butyl; NPPC appears to be the least effective inhibitor of IgG1 and IgG2a antibodies. The pattern of inhibition shown in Figure 4 for day 35 immune serum antibody (Soman-protein >> APSo/NPSo > NPDBP > DPMP > NPPC) was demonstrable at all timepoints tested as indicated by the I50 values provided in Table I.

In terms of fine structure recognition it appears that the phenyl linkage is important in the binding of Soman-specific IgG serum antibody as was found to be the case for Soman-specific monoclonals (2,3,19,20) and polyclonal rabbit antisera (19). The generation of antibodies with specificity which includes recognition of linkage structures has been described for several protein conjugates (21-23) and has been a concern in the construction of protein-coupled vaccines. The presence of the phenyl structure is also required for recognition by memory Group II antibodies generated against phenyl-protein-linked PC (4-7). It appears that antibodies produced in response to So-KLH resemble those generated in the memory response to PC-KLH in diversity of fine specificity and heterogeneous V gene usage. However, in the anti-Soman-KLH response this molecular heterogeneity apparently extends to the primary response as well (3).

The inability to inhibit Soman-protein binding by PC-BSA and its hapten analog NPPC illustrates the lack of PC-phenyl-reactive antibody in the anti-Soman-KLH response and the positively-charged choline moiety of PC is implicated as playing a significant role in preventing this recognition. The

presence of the negatively charged phosphate in PC-BSA and NPPC may be less significant in preventing cross-reactivity since partial inhibition of Soman-specific IgG antibody by NPDBP could be demonstrated despite the presence of a phosphate residue in the latter (Fig. 4, Table I). Also, NPMPC, a methylphosphonate analog of NPPC, was found to be a very poor inhibitor of Soman-specific IgG monoclonal antibodies (Table I & II and data not shown).

Antibody maturation in the anti-Soman-KLH response:

Figure 5 demonstrates that shifts in fine specificity can be observed in anti-So-KLH IgG populations. Both IgG1 and IgG2a serum antibodies showed increased sensitivity to inhibition by Soman-protein with time after immunization. In contrast, inhibition by NPSo did not appear to change significantly. Finally, inhibition by NPDBP and NPPC became less efficient with time. These temporal alterations in recognition are consistent with maturation of the Soman-specific serum antibody response towards recognition of the immunogen So-KLH. In addition, comparison of inhibition values obtained in the late primary/early secondary response to those measured on comparable days after memory boosting reveals a divergence in fine specificity maturation among IgM and IgG1 serum antibodies (Table I).

Soman-protein inhibition of serum IgM 21 days after primary or memory boosting does not differ significantly whereas Soman-protein inhibition of serum IgG1 is 5 to 10-fold greater in the day 14 memory response compared to day 14 after primary immunization. Since the relative avidity, kinetics and levels of IgM antibody produced after memory boosting are similar to those of the primary IgM response, it suggests that the majority of IgM produced to subsequent challenges with Soman-KLH represents recruitment of new

primary B cells into the onging response even after the response is well-established, as seen in other systems (18). Whether primary response IgM and secondary response IgG antibodies derive from the same B cell population or whether they represent distinct B cell populations is not known. IgG1 serum antibodies produced after final boosting are likely to represent products of an antigen-selected memory B cell population (9,10,24).

Interestingly, NPSo and APSo inhibition of IgG1 serum antibody do not appear to vary significantly throughout the entire response to Soman-KLH. The lack of maturation in relative affinity for NPSo/APSo suggests that there are carrier influences or carrier-specific contacts playing a selective role in maturation of the anti-Soman-KLH response. Such carrier-mediated effects on antibody maturation have been suggested by others (25,26). However, since no overall change is apparent in the ability of NPSo/APSo to inhibit serum antibody, NPSo/APSo-binding antibody appears to be maintained as part of the Soman-specific antibody pool. This is in contrast to the temporal decrease in ability of NPPC and NPDBP to inhibit suggesting that as the ability to bind Soman-protein improves, the ability of these related but distinct structures to inhibit decreases since they lack the dominant hydrophobic features of Soman.

Fine specificity of anti-Soman KLH monoclonals:

Table II shows the fine specificity profile of several monoclonals obtained after secondary and tertiary boosting with Soman-KLH. A comparison of Table II, which provides the I_{50} values of various inhibitors obtained for individual monoclonals, with Table I, which provides similar information for serum antibody generated in response to Soman-KLH,

demonstrates that the fine specificity of monoclonal antibody correlates well with what has been observed in Soman-specific serum antibody populations. Although there is significant variation between monoclonals in relative avidity, the overall pattern of specificity appears to be: Soman-protein >> APSo ≥ NPSo ≥ NPDBP >NPPC. It is also noteworthy that 3/4 monoclonal antibodies obtained from a later fusion (tertiary response) showed no inhibition by NPDBP or NPPC. Also, 10/13 IgM monoclonal antibodies were inhibited by Soman-protein only, and were not inhibited by any Soman hapten tested. The analysis of hybridomas obtained after Soman-KLH priming and boosting is therefore likely to be representative of the in vivo Soman-KLH response. We have previously demonstrated heterogeneous V gene usage among Soman-specific hybridomas (3) and this is likely to be true for anti-Soman antibodies produced in vivo as well. The apparent lack of a restricted B cell response at any point in the Soman-KLH response may reflect the absence of idiotypic regulation or evolutionary selection for an environmentally common structure such as PC. Thus, the differences in molecular heterogeneity that characterizes the early phases of the responses to these two organophosphorus haptens may suggest the fact that only one of them, Soman, is new to the environment.

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Table I: mM I50 values for So-KLH-specific serum antibody

serum IgM inhibitor	day7	day14	day21	day35	day122	day290
DPMP	>10	>10	>10	>10		>10
NPSo	>10	>10	>10	>10		>10
NPDBP	>10	>10	>10	>10		>10
NPPC	>10	>10	>10	>10		>10
SoBSA	>.1	>.1	.00061	.015		>.1
SoKLH	.0000082	.00052	.0000061	.00001		.0037
SoOva	.00009	.0066	.000046	.0008		>.1
PCBSA	>.1	>.1	>.1	>.1		>.1
KLH	>1	>1	>1	>1		>1

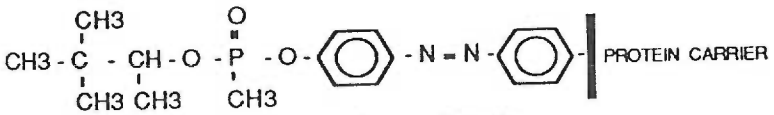
serum IgG1 inhibitor						
DPMP	>10	9.2	9.65	>10	>10	
APSO	.0027	.0048	.0046	.0043	.0027	.001
NPSo	.033	.049	.066	.051	.050	.048
NPDBP	.59	.46	2.3	2.2	2.4	.95
NPPC	1.39	4.11	>10	>10	>10	>10
So-BSA	.00052	.00053	.00046	.00032	.00010	
So-KLH	.000015	.000023	.000011	.000014	.000004	.000002

serum IgG2a inhibitor						
DPMP	6.6	>10	>10	>10	>10	>10
NPSo	.019	.027	.04	.057	.05	.034
APSO	.0027	.0067	.0085	.014	.0073	.0076
NPDBP	.91	2.5	8.0	>10	>10	>10
NPPC	9.9	>10	>10	>10	>10	>10
SoBSA	.000088	.00015	.00006	.000055	.000011	.00002
SoKLH	.0000057	.0000079	.0000024	.0000019	.00000065	.0000012

Table II: Fine Specificity Profiles of 2° and 3° response anti-Soman-KLH hybridomas

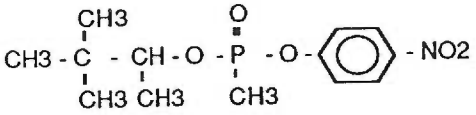
	Inhibitors:							
	APSo	NPSo	NPDBP	NPPC	NPMPC	NPEP	OHSo	SoBSA
2° response IgG1 hybridomas:								
SoG1-3	0.009	0.26	0.388	>10	>10	>10	3.8	8.0 x 10-8
SoG1-5	0.0003	0.002	0.064	9.9	>10	2.98	8.0	5.0 x 10-7
SoG1-9	0.024	0.13	0.053	>10	>10	1.57	1.07	1.7 x 10-7
SoG1-13	0.0003	0.26	0.365	>10	>10	3.47	7.73	3.1 x 10-7
2° response IgG2a hybridomas:								
SoG2a-3	0.001	0.015	0.018	1.02	0.63	2.18	1.0	1.4 x 10-7
SoG2a-4	0.0004	0.02	0.014	0.58	0.63	1.05	0.9	3.0 x 10-7
SoG2a-6	0.0009	0.026	0.025	0.75	0.88	2.15	0.5	9.6 x 10-9
SoG2a-7	0.04	0.18	0.129	3.76	>10	0.58	>10	8.1 x 10-9
3° response hybridomas:								
SoG1-1	0.002	0.0004	0.151	5.64	ND	3.29		4.0 x 10-8
SoG2a-1	0.033	0.033	>10	>10	>10	>10	>10	1.1 x 10-8
SoG2a-2	0.018	0.0008	>10	>10	>10	>10	>10	1.4 x 10-8
SoG2b-1	0.02	0.035	>10	>10	>10	4.03	>10	3.6 x 10-9

Figure 1: Chemical structure of hapten inhibitors.

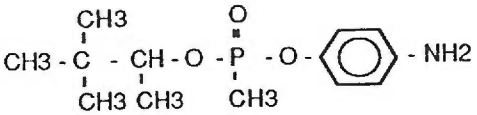


Soman-Protein

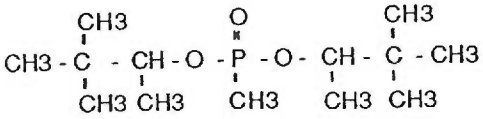
SOMAN DERIVATIVES



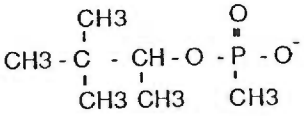
NO2-ϕ-So



NH2-ϕ-So

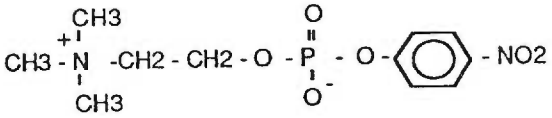


DPMP

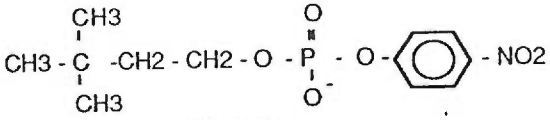


OH-So

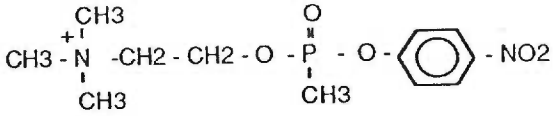
PC DERIVATIVES



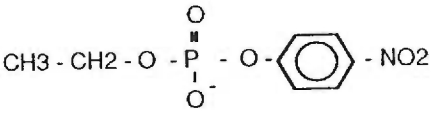
NPPC



NPDBP



NPMPC



NPEP

Figure 2: Serum immunoglobulin response to Soman-KLH immunization.

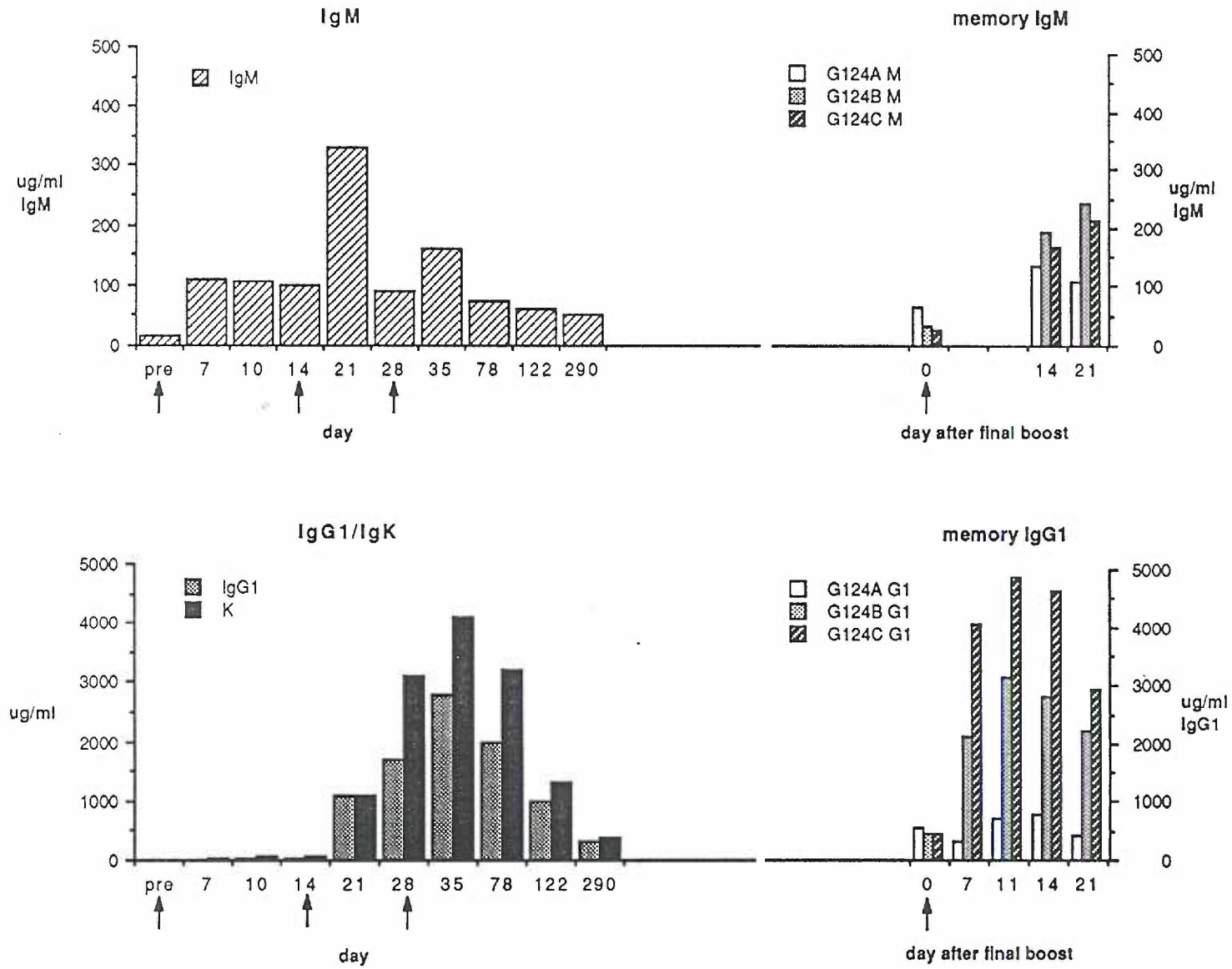

IgM
IgM
ug/ml IgM
500
400
300
200
100
0
pre 7 10 14 21 28 35 78 122 290
day
memory IgM
G124A M
G124B M
G124C M
0 14 21
day after final boost
ug/ml IgM
IgG1/IgK
IgG1
K
ug/ml
5000
4000
3000
2000
1000
0
pre 7 10 14 21 28 35 78 122 290
day
memory IgG1
G124A G1
G124B G1
G124C G1
0 7 11 14 21
day after final boost
ug/ml IgG1

Figure 3: Fine specificity of day 21 serum IgM.

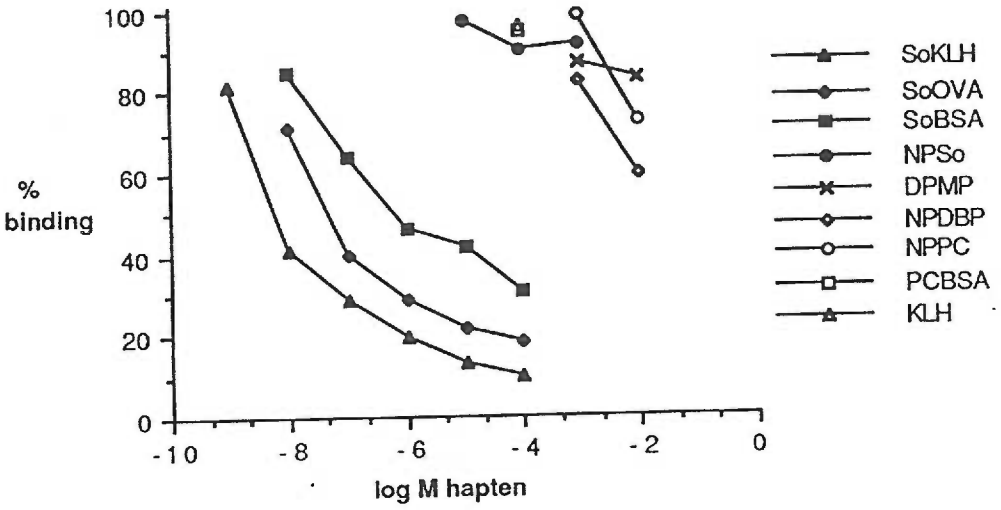

100
80
60
40
20
0
% binding
-10
-8
-6
-4
-2
0
log M hapten
SoKLH
SoOVA
SoBSA
NPSo
DPMP
NPDBP
NPPC
PCBSA
KLH

Figure 4: Fine specificity of day 35 anti-Soman-KLH serum IgG1 and IgG2a.

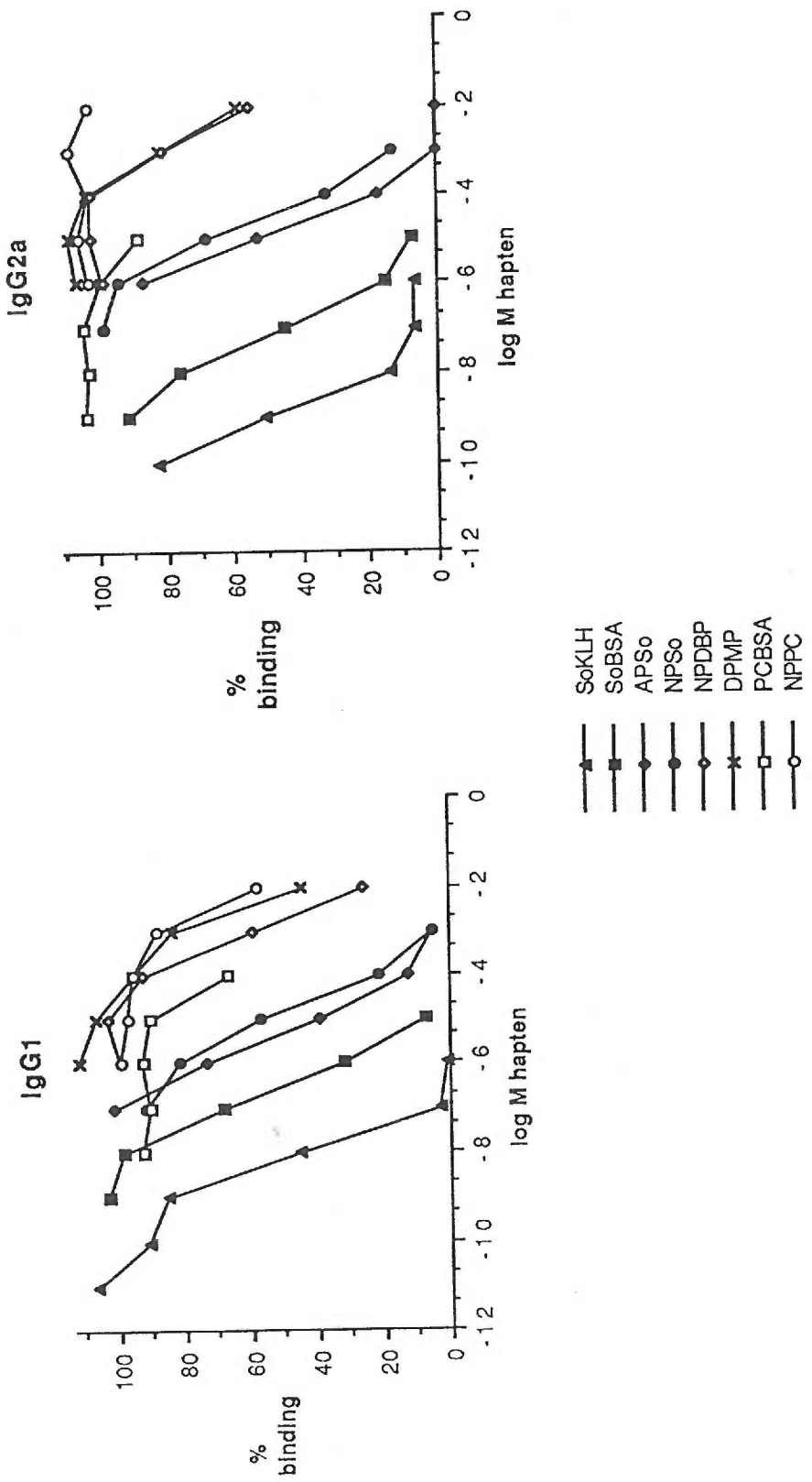
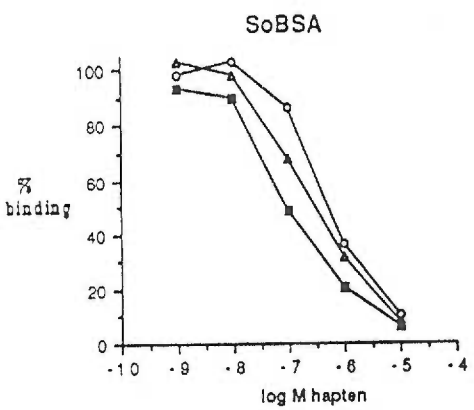
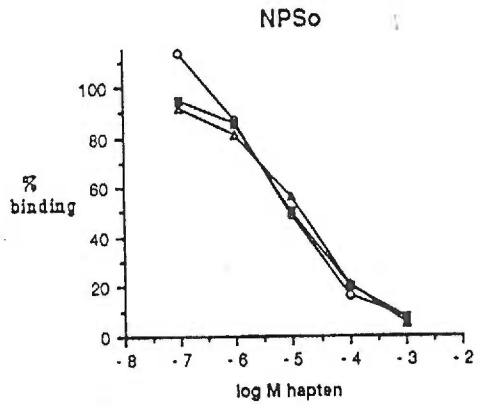

IgG2a
IgG1
% binding
log M hapten
SoKLH
SoBSA
APSo
NPSo
NPDBP
DPMP
PCBSA
NPPC

Figure 5: Maturation of fine specificity in response to Soman-KLH. Statistical significance was analyzed using the student T-test and compared the I_{50} values (5-6 independent measurements) of one serum πsample against that of others.

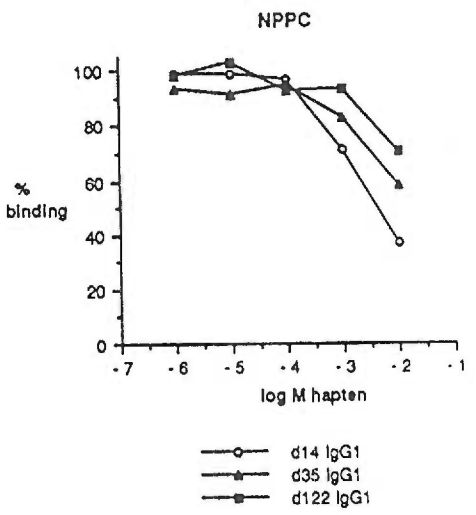
A. Serum IgG1



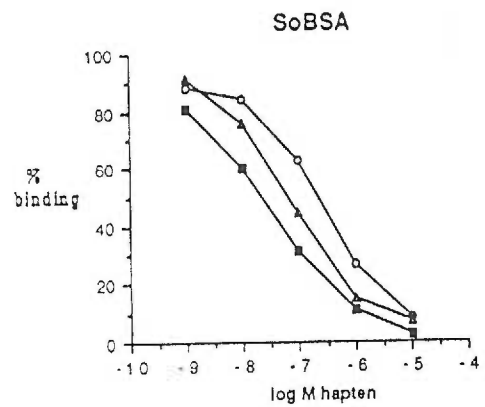
d14 vs d35, $p < 0.3$
d35 vs d122, $p < 0.2$
d14 vs d122, $p < 0.01$



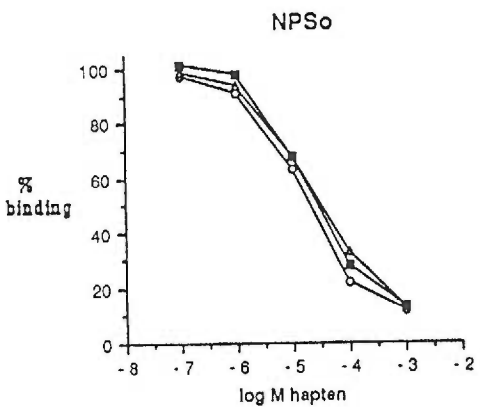
d14 vs d35, $p < 0.3$
d35 vs d122, $p < 0.4$
d14 vs d122, $p > 0.9$



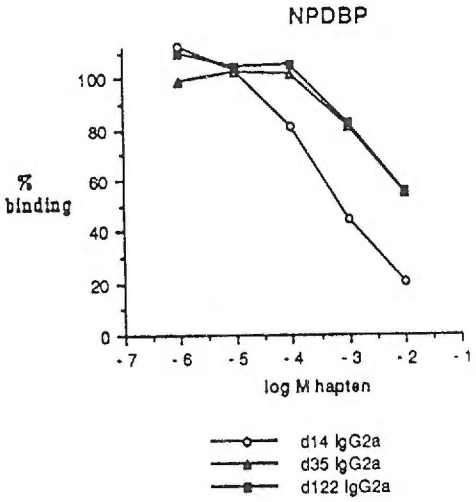
B. Serum IgG2a



d14 vs d35, $p < 0.05$
d35 vs d122, $p < 0.05$
d14 vs d122, $p < 0.02$



d14 vs d35, $p < 0.5$
d35 vs d122, $p < 0.9$
d14 vs d122, $p < 0.6$



SUMMARY AND DISCUSSION

The antibody response to Soman-KLH is clearly and consistently heterogeneous. Diversity extends from the level of V gene usage, particularly that of the VH, to the proposed structure of the combining site itself. The observed result is overall heterogeneity in the fine specificity of functional recognition.

Fine specificity analysis of anti-Soman-KLH antibodies defined the phenyl region as playing a dominant role in recognition. The finding that the same phenyl structure is important in the binding of anti-PC-KLH antibodies (1,2,3) suggests that such a structure may generally be an important, perhaps stabilizing feature in molecular recognition. In fact, several well-studied hapten systems include aromatic structures such as *p*-azophenylarsonate (4), phenyloxazolone (5), nitrophenylacetyl (6), fluorescein (7), and phthalate (8).

Despite the apparent diversity, the response to Soman-KLH remains highly specific, accomplishing one of the most important tasks of the immune response. In contrast to binding of anti-PC-KLH antibodies, presence of a positively charged choline is detrimental to the binding of anti-So-KLH antibodies, thus ensuring that cross-reactivity is at a minimum despite significant recognition of the phenyl by both anti-So-KLH and anti-PC-KLH antibodies. Charge associated with the phosphonate structure appears to be less influential for the binding of anti-So-KLH antibodies. By defining regions of the antigen most important in the recognition of specific antibodies, we have identified regions most likely to play a role in antigen selection. However, an interesting contrast in functional contribution of the phenyl vs. the charged (PC) or uncharged (Soman) terminal structures is observed. Since a similar phenyl structure is a major contributor to binding

in both antibody responses, it is unlikely that recognition of the phenyl alone defines the non-cross-reactivity between antibodies specific for So-KLH and PC-KLH. On the other hand, recognition of the charged (anti-PC-KLH) or uncharged (anti-So-KLH) terminal structure is not only required for binding but may also defines this non-cross-reactivity. Whether these functions correspond to positional distribution is unclear, although Novotny et al. (32) have suggested that binding energy in three known Fab-ligand complexes originates mainly from interactions centered at the bottom of the combining site. The relative contributions of these functionally distinct regions to antigen selection remains to be elucidated.

These studies do not directly address the nature or extent of contribution by carrier detemrinants to specific recognition or selection. However, as with the response to PC-KLH, temporal maturation of the So-KLH-specific IgG population towards better recognition of Soman-protein conjugates and the greater than 10^3-fold difference in inhibition by free Soman analogs and Soman-protein conjugates are highly suggestive of carrier influences. In the x-ray structure of M603, bound PC occupies only a small region at the base of the combining site (9) compared to the extensive surface contact observed between lysozyme and lysozyme-specific Fabs (10,11). It would seem logical, therefore, that carrier determinants of So-KLH make some contact with the combining site. Claflin et al. (12) have proposed that variable residues lining the outer surface of the combining site of PC-binding proteins may play a significant role in distinguishing between PC associated with different microorganisms. Such residues would be in a position to contact carrier residues surrounding the coupled Soman hapten.

Unlike the IgG antibodies generated in response to So-KLH, IgM antibodies showed little or no inhibition by Soman hapten analogs and,

therefore, remain poorly defined in terms of molecular recognition. Inhibition by Soman-protein but not by control protein conjugates or protein alone established, however, that these IgM antibodies were indeed Soman-specific. IgM- and IgG-producing hybridomas used very similar Vk genes and VH genes derived from the same families. Moreover, IgM Vk's showed little mutation (one out of three showed any mutation at all) relative to that seen in the IgG Vk's. It would seem reasonable, therefore, that the IgG-producing clones derive from the non-hapten-inhibitable, IgM-producing precursors which must undergo somatic mutation and class switching in the course of selection by So-KLH and thereby gain inhibitability by hapten.

The possibility also exists that anti-So-KLH IgM- and IgG-producing B cell clones derive from different B cell populations. Naturally occurring, poly-reactive IgM antibodies are present at high levels in normal serum (13,14) and appear to be encoded by V genes used in antigen-specific responses (15). Such antibodies may play a role in natural immunity (16) providing sufficient protection during initial exposure to pathogen in the absence of highly specific antibodies. The B cells producing these antibodies may belong to a distinct subset of B lymphocytes expressing the Ly1 marker (17,18) and may follow a pathway distinct from that of antigen selected B cells. Therefore IgM antibodies detected in the early anti-So-KLH response could arise from this alternate set of B cells while precursors of the IgG-producing clones, presumably low in frequency, are necessarily undergoing selection and clonal expansion.

The interpretation of changes associated with maturation of the antibody response is complicated by the fact that relatively little is known concerning the compartmentalization of B cell function. Consistent with the observation that newly arising B cells are continually recruited into established responses

(19) and that this recruitment is ongoing in the secondary lymphoid organs (20), splenic fusion after secondary and tertiary boosts with So-KLH gave rise to IgM-producing hybridomas with characteristics of primary IgM clones. The anti-So-KLH IgM serum response following memory boost also showed antibody levels and kinetics similar to that of a primary response. The finding that most plasma cells arising from memory B cells migrate to the bone marrow (20) may mean that conventional splenic fusions do not sample much of the fully matured, specific B cell population. With this in mind, an analysis of affinity and fine specificity differences and of the somatic mutations incurred by antibody-producing, bone marrow-derived B cell clones could prove to be highly informative regarding the continued maturation of a specific antibody response.

The significance of frequent Vk1 family usage in the anti-So-KLH response as well as the anti-PC-KLH response is unclear. The predominance of Vk1 usage in antibody responses involving phenyl- or ring-containing structures has been noted (21,22) and may imply that certain V elements are associated with recognition of particular structural features. On the other hand, Kaushik et al. (23) observed that the Vk1 family, while not very large, was expressed at a disproportionately high frequency in mitogen-stimulated splenic B cells. Others have also suggested that the Vk1 family contributes to the pool of naturally occurring multireactive antibodies (24).

The intriguing preference for use of Vk1A in anti-So-KLH antibodies and use of Vk1C in the anti-PC-KLH response (22,25) calls for some degree of specificity on the part of these highly homologous genes (26) in the respective responses. Such specificity may be provided by the charge difference observed at position L34 at which the presence of Glu in Vk1C may be more conducive to binding of the positively charged nitrogen of PC

whereas for the binding of Soman, the presence of His in Vk1A may be preferred. Alternatively, a difference in the packing of VL against VH as a result of differing interface residues present in Vk1A vs. Vk1C may alter the conformation of the combining site sufficiently to be preferential for one but not the other hapten. Yet a third possibility may be that differential interface residues limit association of VL with a certain set of VH thereby creating a restriction in specificity. Multiple VH gene families encode both anti-So-KLH and anti-PC-KLH antibodies (22,25). However, whereas the J558 family was found to be used by the majority of anti-So-KLH antibodies (>75%), this large family was poorly represented among anti-PC-KLH antibodies (22,25). In contrast, the Q52 family appears to contribute significantly to the PC-KLH (22,32) response while Q52 was barely represented among anti-So-KLH antibodies. This observation of differential VH preference correlates with the preferential use of Vk1A over Vk1C in the anti-So-KLH response and vice versa in the anti-PC-KLH response.

Light chains of anti-So-KLH antibodies display a very low frequency of mutation associated with both framework and CDR residues. The low R:S ratio (8:10 = 0.8) of the CDRs may signify that these germline sequences are required for functional binding and have been negatively selected for replacement (27). This would imply that Vk1 light chain-derived combining site residues require little change for the optimal binding of So-KLH. Consistent with preference for Vk1A in anti-So-KLH hybridomas, the single Vk1C gene used by an anti-So-KLH hybridoma (SoG2b-1) possesses a relatively high proportion of replacement mutations in CDR3. Interestingly, the Vk1C light chains of anti-PC-KLH antibodies have low R:S values (25) as if in that response they, similar to the Vk1A light chains of anti-So-KLH antibodies, do not tolerate many replacements.

On the other hand, the frequency of mutation associated with certain VL has been found to be disproportionate in comparison to their respective VH (28) and may indicate that not only does the mutational mechanism operate unequally on VH and VL, but that somatic mutation may be applied unequally to different VL. In the anti-PC-KLH response, a high frequency of somatic mutation was found associated with Vλ but not with Vk1 light chains (25). Vk1 may therefore be relatively resistant to mutation. Conceivably, if VH contributes most of the Soman contact residues as observed in the case of M603 and bound PC (29), the non-mutability of Vk1 may not significantly impede selection for higher affinity variant clones.

The heterogeneity of antibodies generated in response to So-KLH may be representative of a situation in which the immune system encounters a novel antigenic determinant. That is, the absence of an evolutionarily fixed response to So-KLH necessitates the recruitment of a heterogeneous pool of B cell clones which are submitted to antigenic selection. The result is a matured but heterogeneous population with increased specificity for So-KLH. This is in direct contrast to restricted antibody responses against pathogen-associated determinants such as PC, which may have evolved as a result of selective advantage (30,31). The observed shift from a restricted primary to a heterogeneous secondary response to PC-KLH may be complicated because of similarity to the environmental antigen. However, consistent with the theme of antigen selection, the mature antibody population in the anti-PC-KLH response has increased specificity for the immunogen, PC-protein (22). The function of heterogeneity as typified by the anti-So-KLH response described here, although seemingly basic, is most likely to be an important mechanism for survival against newly arising or infrequently encountered pathogens.

In conclusion, the work presented here provides the necessary ground work for continued research on the structural basis for antibody specificity and the process of antigen selection in the anti-Soman-KLH response. The proposed models of the anti-Soman-KLH and anti-PC-KLH combining sites allow the formulation of specific questions concerning the relationship of antibody structure to functional recognition. With the availability of modeling techniques and modern molecular tools, i. e. site-directed mutagenesis and Ig chain recombination, such questions may be readily answered. In doing so, this information may be added to our rapidly growing knowledge of combining site interaction at the molecular level. Studies of antibody structure-function relationship have already made significant advances in the use of antibodies as tools for medical therapy and biotechnology. These include production of antibodies as idiotypic vaccines (34), design of antibodies with catalytic activity (35,36), and, most recently, the isolation of CDR residues as biologically active peptides (37). Such success holds great hope for the future of immunologic research.

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